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THIORHODACEAE

I. THE EFFECTS OF SODIUM THIOGLYCOLATE ON THE PHOTOSYNTHETIC AND DARK METABOLISM OF PURPLE SULPHUR BACTERIA[1]

JOHN J. TAYLOR

Abstract

Twenty-two pure cultures of purple sulphur bacteria were investigated for their ability to utilize organic sulphur compounds as hydrogen donors during photosynthesis. Of the 12 compounds tested, only sodium thioglycolate sustained growth of the organisms in the light.

Carbon dioxide fixation by illuminated resting cells in the presence of endogenous sulphur or exogenous thiosulphate was inhibited by thioglycolate in proportion to its concentration. The fixation of carbon dioxide by illuminated sulphur-free resting cells harvested from thioglycolate media increased with the thioglycolate concentration to about 0.04 *M*, but was proportionally inhibited by higher concentrations.

Non-illuminated suspensions of resting cells in the presence of endogenous sulphur or exogenous thiosulphate also assimilated carbon dioxide when thioglycolate was added. Inhibition of fixation appeared similar to that occurring in the light. Thioglycolate disappeared from the suspensions in proportion to the amount of bicarbonate assimilated. Thioglycolate-grown cells did not fix significant amounts of carbon dioxide under similar experimental conditions. Growth of the organisms was never demonstrated in non-illuminated thioglycolate media.

Introduction

The purple sulphur bacteria have long been known to dehydrogenate sulphide, sulphite, and thiosulphate to sulphate to supply hydrogen necessary for the reduction of hydroxyl ions formed during the photosynthetic process (15). Muller (8) reported that acetate, propionate, succinate, fumarate, malate, lactate, pyruvate, and butyrate also function as hydrogen donors in illuminated cultures. Van Niel (16) concluded that these simple organic acids were completely dehydrogenated to carbon dioxide and water, although some intermediary compounds may have been assimilated by the cells.

Van Niel also pointed out (15) that the sulphur bacteria are found in waters containing hydrogen sulphide, which is porbably the chief hydrogen donor utilized by these organisms in nature. But mercaptans and other thio compounds may also be expected to appear during the anaerobic decomposition of organic matter in nature. Since many of such compounds resemble

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Contribution from the Department of Bacteriology, Montana State University, Missoula, Montana, U.S.A.

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chemically both the simple organic acids and hydrogen sulphide, they may also be expected to serve as hydrogen donors during photosynthesis. However, investigations determining the effects of organic sulphur compounds on the purple sulphur bacteria have never been reported in the literature.

Endogenous fermentation in non-illuminated *Chromatium* species strain D resulted in the production of carbon dioxide, acetic acid, and either hydrogen sulphide or molecular hydrogen (5). Nakamura (9) reported the production of molecular hydrogen during the anaerobic decomposition of formate, pyruvate, glucose, glycerol, and glycerophosphate by non-illuminated suspensions of *Chromatium minutissimum*. Since the "normal" photosynthetic hydrogen donors have been found to have no effect on those organisms previously examined, it seemed advisable to test the organic sulphur compounds under these conditions also.

Materials and Methods

Samples of mud and water were taken from ponds and marshes on Pelee Island, Ontario, and the Bass Islands in western Lake Erie. Enrichment and pure cultures were prepared according to the methods of van Niel (15). The medium used was that of Larsen (6) and Newton and Wilson (10) and had the following percentage composition: ammonium chloride, 0.1; monohydrous monobasic potassium phosphate, 0.1; hexahydrous magnesium chloride, 0.05; agar (Difco), 1.5; sodium bicarbonate, 0.2; and nonahydrous sodium sulphide, 0.1. For the screening tests, sodium sulphide was omitted and an organic sulphur compound which contained an equivalent amount of sulphur was added. All stock cultures were prepared in 1-oz prescription bottles sealed with screw caps, and were incubated at room temperature (about 27° C) at a distance of about 50 cm from a 150-w reflector-type incandescent lamp.

Cells for manometric determinations were harvested by centrifugation at 5° C from 96-hour agar-free cultures, and were washed three times with 0.01 *M* pH 7.4 phosphate buffer prior to use. Exposure to atmospheric oxygen did not alter cellular activity (10). Carbon dioxide uptake was measured at 25° C with a conventional Warburg respirometer. Double sidearm flasks were used throughout. The main compartment contained 0.5 ml of a 10% suspension by volume of washed cells in pH 7.2 phosphate buffer, and 0.5 ml of 0.033 *M* sodium bicarbonate solution which maintained pH 7.2 during the experiment. One sidearm contained 0.5 ml of substrate in pH 7.2 buffer, or buffer alone as a control. The second sidearm contained 0.5 ml of 3 *N* sulphuric acid. All flasks were initially gassed with a mixture of 5% carbon dioxide in nitrogen, then closed and illuminated by two 100-w incandescent bulbs located about 30 cm above the water bath. When necessary, the vessels were darkened either by wrapping the individual flasks with aluminum foil, or by covering the entire water bath with heavy black cloth. Bicarbonate exchange during the experimental period was calculated by the method of Umbreit, Burris, and Stauffer (14).

Compounds containing the sulphydryl radical were determined quantitatively by the method of Kimball, Kramer, and Reid as modified by Siggia (12). If both sulphide and thioglycolate occurred in the same sample and both were to be measured, an aliquot was initially titrated, then the remainder was boiled at pH 3.0, cooled, adjusted to pH 7.0, restored to original volume, and titrated again. The titration before boiling included both sulphide and thioglycolate; the titration after boiling measured thioglycolate alone. The difference between the titrations represented the decrease in iodine equivalent to the sulphide lost in boiling (5). When thioglycolate alone was to be determined, all samples were boiled at pH 3.0 before titration.

Results

Effects of Sulphur Compounds on the Growth of Purple Sulphur Bacteria

The results of the screening tests are presented in Table I. Only 8 of the 22 cultures examined grew in media containing thioglycolate, the only organic sulphur compound tested which supported normal growth. Otherwise, all strains responded quite uniformly to the various substrates added.

In order to confirm the observation of growth in thioglycolate medium and to rule out the possibility of contamination, tubes of brain–heart infusion broth (Difco) were inoculated from the original thioglycolate cultures and incubated aerobically and anaerobically in the dark. No growth was evident in these tubes even after 20 days at room temperature. Organisms from the thioglycolate cultures were also passed through three serial thiosulphate agar shake tube cultures and reisolated. When the organisms were returned to thioglycolate medium, growth again occurred. Four serial transfers through thioglycolate medium yielded growth within 3 to 5 days after each transfer.

TABLE I

SULPHUR COMPOUNDS USED FOR SCREENING TESTS*

Compounds supporting growth	Concn. (g/l.)	Compounds not supporting growth	Concn. (g/l.)
Sulphide ($S^{=}$)	1.00	Sulphite ($SO_3^{=}$)	1.10
Thiosulphate ($S_2O_3^{=}$)	1.00	Bisulphite (HSO_3^{-})	0.90
Dithionate ($S_2O_6^{=}$)	2.10	Metabisulphite ($S_2O_5^{=}$)	1.58
Sulphydrate (SH^{-})	0.47	Pyrosulphate ($S_2O_7^{=}$)	1.93
		Thiocyanate (SCN^{-})	0.68
Thioglycolate	0.96	Ethyl xanthate (K salt)	1.35
		Methyl bisulphide	0.79
		Thioacetate	0.64
Simple organic acids		Thioacetamide	0.63
		Thiourea	0.64
Acetate	0.50	Thiouracil	1.10
Propionate	1.00	Thiomalate	1.29
Succinate	1.00	Cysteine	2.80
Malate	1.00	Cystine	2.03

*All salts were sodium salts unless otherwise specified.

A series of five tube cultures, containing thioglycolate in concentrations from 0.01 to 0.15 *M*, was prepared in duplicate and inoculated. Both series were incubated anaerobically at room temperature, one series in the light, the duplicate series in the dark. Growth occurred within 3 days in illuminated cultures containing 0.01 or 0.02 *M* thioglycolate. No growth was apparent at higher concentrations. None of the cultures incubated in the dark gave evidence of growth.

Certain sulphur-free acids which may have been intermediates in thioglycolate utilization were examined. Growth never occurred in media containing from 0.01 to 0.3% sodium glycolate regardless of illumination. Media containing 0.1% concentrations of acetic, propionic, succinic, or malic acid supported growth in the light but not in the dark.

Effects of Thioglycolate upon Carbon Dioxide Assimilation by Illuminated Suspensions

Illuminated suspensions of cells containing intracellular sulphur granules fixed carbon dioxide at an average rate of 45 μl per hour. When sodium thioglycolate, pH 7.2, was added to the suspensions, the rate of carbon dioxide assimilation was reduced. The inhibitory effect of thioglycolate increased in proportion to its concentration (Fig. 1). When the molarity of the thioglycolate added reached 0.3 *M*, carbon dioxide uptake ceased. No significant change in the thioglycolate concentration in the suspensions could be detected by titration.

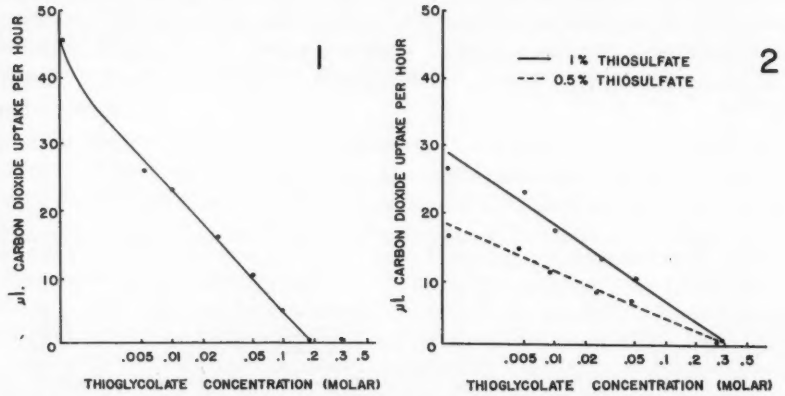

FIG. 1. Thioglycolate inhibition of carbon dioxide uptake in illuminated suspensions of sulphide-grown cells containing endogenous elementary sulphur.
FIG. 2. Thioglycolate inhibition of carbon dioxide uptake in illuminated suspensions of thiosulphate-grown cells supplied with exogenous thiosulphate.

An inhibition of bicarbonate assimilation almost identical with that produced by thioglycolate was observed when equimolar concentrations of sodium glycolate (glycolic acid adjusted to pH 7.2 with 10% sodium hydroxide) replaced thioglycolate. It seemed possible that thioglycolate could

have been interfering with the oxidation of sulphur, or, by structural similarity, with glycolaldehyde, a suggested acceptor for carbon dioxide (2). But the inhibitory effect of thioglycolate was not suppressed by sulphide added to a concentration of 0.002 *M* or 0.01 *M* glycolaldehyde.

Cells harvested from 0.1% sodium thiosulphate or from 0.01 *M* sodium thioglycolate media never contained sulphur granules. When illuminated, suspensions of these cells did not fix carbon dioxide unless a sulphur compound was present. Thiosulphate-grown cells assimilated carbon dioxide at an average rate of 24.6 μl per hour when they were suspended in 0.1% sodium thiosulphate. In 0.05% thiosulphate, the cells assimilated an average of 17.5 μl of carbon dioxide per hour. When, in addition, thioglycolate was added to the suspensions, the rate of bicarbonate assimilation again decreased nearly proportional to the concentration of thioglycolate added (Fig. 2). Sulphur-free, thiosulphate-grown cells did not fix carbon dioxide in the presence of thioglycolate alone.

When increasing amounts of thioglycolate were added to illuminated suspensions of sulphur-free thioglycolate-grown cells, carbon dioxide was assimilated (Fig. 3). The rate of carbon dioxide uptake increased in proportion to the thioglycolate concentration until about 0.025 *M* had been added. With greater concentrations of thioglycolate, carbon dioxide fixation decreased, but was still evident at 0.05 and 0.10 *M* even though growth in these concentrations never occurred.

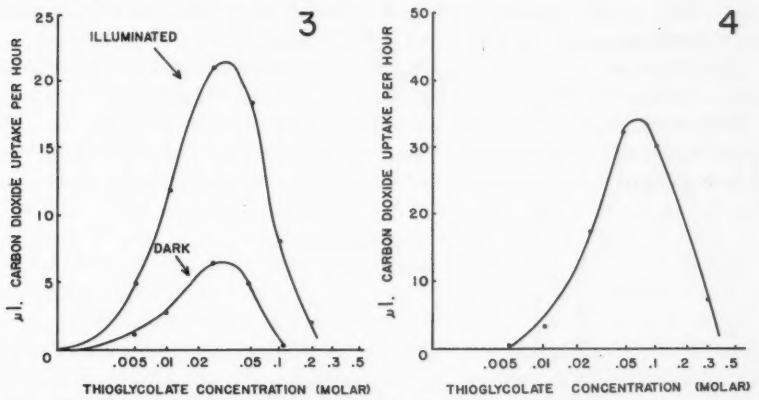

FIG. 3. Carbon dioxide uptake by thioglycolate-grown cells suspended in thioglycolate.

FIG. 4. Carbon dioxide uptake in the presence of thioglycolate by non-illuminated cells containing endogenous elementary sulphur.

Effects of Thioglycolate upon Carbon Dioxide Assimilation by Non-illuminated Suspensions

In the dark, uspensions of sulphide-grown cells containing intracellular sulphur did not fix carbon dioxide. In fact, there was usually an increase of about 15 μl in the total carbon dioxide in the darkened flasks after 4 hours. When thioglycolate was added to the non-illuminated suspensions,

carbonate disappeared (Fig. 4). The rate of carbon dioxide uptake increased with increasing concentrations of thioglycolate up to about 0.07 *M*. At higher concentrations, the rate of uptake decreased rapidly. Inhibition of the dark metabolism was nearly complete with 0.3 *M* thioglycolate. Titrimetric analyses of those suspensions in which there had been maximum carbonate assimilation in the dark revealed that 1 mole of thioglycolate disappeared for every 3.5 to 4.0 moles of carbon dioxide assimilated.

When equimolar concentrations of glycolate were substituted for thioglycolate, no carbon dioxide uptake occurred. This result was in agreement with the inability of the organisms to grow in glycolate media.

There was no uptake of carbon dioxide in non-illuminated suspensions of sulphide-grown cells to which 0.002 *M* sulphide alone had been added. But this concentration of sulphide completely inhibited the dark metabolism of thioglycolate by similar suspensions. Likewise, the dark assimilation, as measured by carbon dioxide uptake, was almost completely inhibited by 0.01 *M* glycolaldehyde.

Neither thiosulphate- nor thioglycolate-grown cells assimilated carbon dioxide in the dark in the absence of sulphur compounds. Nor could an uptake of carbon dioxide be observed when various concentrations of thioglycolate were added to suspensions of thiosulphate-grown cells.

However, when 0.01 to 0.10 *M* thioglycolate was added to suspensions of thioglycolate-grown cells in the dark, a small amount of carbon dioxide was fixed. The uptake was greatest with about 0.03 to 0.05 *M* thioglycolate, then decreased rapidly to zero with 0.10 *M* (Fig. 3).

Suspensions of thiosulphate-grown cells in 0.1 or 0.05% sodium thiosulphate produced from 5 to 10 μl of carbon dioxide. When thioglycolate was added to these suspensions, there was an uptake of carbon dioxide (Fig. 5). The extent of this uptake was nearly the same as that observed in suspensions of sulphide-grown cells in similar concentrations of thioglycolate.

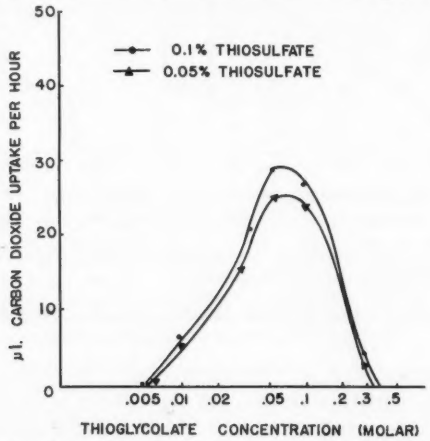

FIG. 5. Carbon dioxide uptake in the presence of thioglycolate by non-illuminated cells supplied with exogenous thiosulphate.

Discussion

Several observations have indicated that the strains of purple sulphur bacteria used in this investigation differ in certain respects from those strains previously described in the literature. First, the organisms did not grow in media containing sulphite, but grew well in dithionate cultures. Second, nearly all strains produced heavy capsules in media containing less than about 60 p.p.m. hydrogen sulphide. Although it is evident from van Niel's experiments (15) that mucus-forming strains were among those which he isolated, detailed work with such organisms has never been reported. Third, cellular activity was not affected by contact of the cells with atmospheric oxygen during normal culturing, harvesting, and suspending procedures. And fourth, and perhaps the most significantly different characteristic of some of these strains is their ability to assimilate carbon dioxide and grow photosynthetically at the expense of thioglycolate.

If one were to modify van Niel's equation for the dehydrogenation of sulphide (17), a more generalized equation for sulphydryl compounds could be written:

$$4CO_2 + 2RSH + 6H_2O \longrightarrow 4(CH_2O) + 2H_2SO_4 + 2ROH.$$

This equation is in agreement with the observed utilization of sulphydrate (NaSH) for growth if sulphydryl ion is not significant in bacterial photosynthesis (15). However, other mechanisms for thioglycolate oxidation must be considered.

The reduction of thioglycolate to acetate and hydrogen sulphide, both of which may be utilized for growth, is unlikely. Not only would the original reduction decrease the net gain of hydrogen, but all the hydrogen resulting from the complete oxidation of acetate would be required for the reassimilation of the carbon dioxide produced during the oxidation. Stoichiometrically, 1 mole of thioglycolate would account for no more than 1.5 moles of carbon dioxide. This is significantly different from the observed values which lay between 3.5 and 4 moles of carbon dioxide per mole of thioglycolate.

The oxidation of thioglycolate to dithioglycolate is also improbable since at least 4 moles of the former would be required for the reduction of 1 mole of carbon dioxide. Furthermore, the disulphide is probably stable to sulphur oxidation since methyl disulphide and cystine, which contain similar sulphur to sulphur bonds, did not permit growth of the organisms in the light.

Thioglycolate is probably not desulphydrated through hydrolysis to glycolic acid and hydrogen sulphide (4). Glycolic acid was not utilized by the organisms for growth, and the oxidation of hydrogen sulphide alone could not have accounted for the total bicarbonate assimilation.

However, a pathway similar to that described by Pirie (11) and Fromageot (3) for the oxidation of cysteine in liver sections seems the most acceptable and would yield glycolic acid and sulphate and a stoichiometry of 2 moles of carbon dioxide per mole of thioglycolate.

Since none of the proposed mechanisms can entirely account for the amount of carbon dioxide assimilated in these experiments, endogenous materials

may also be concomitantly functional. Although several mechanisms of action could be suggested, the most acceptable seems to be a carbon dioxide fixation similar to if not identical with that found in green plants (1). The oxidation of 1 mole of thioglycolate to glycolic acid and sulphate can supply energy sufficient for the phosphorylation of 4 moles of ribulose monophosphate to ribulose diphosphate. This in turn would be sufficient to fix 4 moles of carbon dioxide in phosphoglycerate. The subsequent essential reduction of phosphoglycerate to glyceraldehyde phosphate may explain why the actual observed ratio of carbon dioxide to thioglycolate was generally a little less than 4:1.

Thioglycolate must function as a hydrogen donor also in the dark. Elementary sulphur and thiosulphate appear to act as hydrogen acceptors, thus allowing the creation of high energy phosphate for the fixation of bicarbonate. Cells containing sulphur were more active than sulphur-free cells supplied thiosulphate. This may indicate that (1) normal photosynthetic oxidation of sulphide may not proceed through thiosulphate as an intermediate, or (2) if thiosulphate does arise as an intermediate, the reaction is not completely reversible, or (3) thiosulphate is not as efficient a hydrogen acceptor as sulphide for synthetic reactions.

Since hydrogen donors in bacterial photosynthesis are dehydrogenated by "dark" reactions, the mechanisms of thioglycolate oxidation are probably the same whether the cells are illuminated or not. The major difference would appear to be the hydrogen acceptor, hydroxyl ion in the light, and some endogenous material in the dark.

The most acceptable mechanism already proposed for thioglycolate oxidation would allow fixation of 4 moles of carbon dioxide in phosphoglycerate, but sufficient energy could not be released during the process for the regeneration of carbon dioxide acceptor (ribulose diphosphate) or for the reduction of phosphoglycerate to glyceraldehyde phosphate. Phosphorylysis of capsular mucin may make sufficient pentose phosphate available to the cell for a bicarbonate assimilation independent of the small amount of any other endogenous acceptor.

Since growth did not occur in the dark, the reduction of phosphoglycerate may be thought to be light-dependent. But such a reduction would have yielded a stoichiometric ratio of only 2 moles of carbon dioxide per mole of thioglycolate. Thus, resting cells apparently do not reduce phosphoglycerate even when illuminated. This suggests that the reaction may be growth-linked.

In addition, other reactions necessary for growth may require hydrogen acceptors. But acceptors with a sufficiently high potential, comparable to hydroxyl ion produced by the photolysis of water, may not be available under dark anaerobic conditions.

It is unlikely that thioglycolate inhibited carbon dioxide uptake by direct interference in the normal sulphur oxidations. First, the inhibition occurred equally with both thioglycolate and glycolic acid. Second, the addition of

normal substrate did not overcome the inhibition. These observations do not of course preclude competitive inhibition with a sulphydryl-oxidizing enzyme (7).

Since the inhibitory effect of thioglycolate was observed with both illuminated and non-illuminated suspensions, it probably lay, not in the photodecomposition of water, but in the subsequent reactions leading to the assimilation of carbon dioxide through dark reactions. The recent work of Tolbert (13) indicates that glycolic acid may play an important fundamental role in carbon dioxide fixation, and implies that high concentrations of glycolate (or a structural analogue such as thioglycolate) may inhibit bicarbonate assimilation.

Acknowledgment

The author wishes to express his gratitude to Dr. Chester I. Randles of the Department of Bacteriology, The Ohio State University, for his valuable advice and criticisms during this investigation.

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STAPHYLOCOCCAL TOXINS

II. FACTORS AFFECTING HEMOLYSIS BY δ-LYSIN[1]

A. W. JACKSON AND R. M. LITTLE

Abstract

The influence of a number of variables upon lysis of erythrocytes by staphylococcal δ-lysin has been examined. Hemolysis of human erythrocytes by δ-lysin has been found to be unaffected by the presence of α-lysin but is inhibited by a number of proteins, serum being the most active of those tested. Glucose, citrate, and magnesium ions were found to have no effect on hemolysis by δ-lysin. Hemoglobin and erythrocyte ghosts were inhibitory. Release of cell constituents during hemolysis is considered to be partly responsible for the change from a rapid to a slower rate of hemolysis in the early stages of the reaction.

It was found that the extent of hemolysis of a 1% suspension of human erythrocytes is linearly related to lysin concentration provided lysis is not greater than 65% nor less than 30% of total hemolysis. A method of determining relative δ-lysin activity is described.

Introduction

It is now well recognized (5, 7, 16) that coagulase-positive strains of *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) may produce at least three different hemolysins, designated as α-, β-, and δ-lysins. The latter is characterized by its capacity to hemolyze human erythrocytes (16), which are generally considered resistant to direct lysis by α- and β-lysins (13, 16). In spite of the fact that of the three well-accepted hemolysins δ-lysin alone possesses capacity to cause direct lysis of erythrocytes of human origin, production of this lysin has not been regarded as a significant index of pathogenicity of *S. aureus* cultures. It should be noted, however, that some of the characteristics of this lysin suggest that it may play a role in the infection process. δ-Lysin is produced by a rather large proportion of *S. aureus* cultures isolated from human infections (5, 12) and intradermal injection of partially purified lysin into rabbits produces a dermonecrotic effect similar to, although less intense than, that caused by α-lysin (13). Furthermore, some evidence has been obtained in this laboratory (9) to indicate that δ-lysin may be identical with the human leucocidin described by Panton and Valentine (14). The leucocidal nature of δ-lysin has recently been confirmed by Gladstone and van Heyningen (6). Consequently, it would appear that further work on this lysin is justified in order to obtain a more reliable estimate of its significance in the infection process.

At present a method for the quantitative determination of δ-lysin is required to facilitate purification and production studies. Marks and Vaughan (13) reported the activity of crude and partially purified δ-lysin as that dilution

[1]Manuscript received May 9, 1958.
Contribution from the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

of lysin which in 1 hour caused 50% hemolysis of a 1% suspension of human erythrocytes. They concluded that small amounts of α-lysin in these preparations would not interfere with the determination since it had been shown (13) that α-lysin is, at the most, only feebly lytic toward these cells. This does not eliminate the possibility, however, that α-, in the presence of δ-lysin, may have either synergistic or inhibitory effects on hemolysis by the latter lysin.

The cultures employed in the present study produced α- and δ-lysins but were screened to eliminate those which produce β-lysin, since according to Marks and Vaughan (13) the presence of this lysin in δ-lysin preparations would probably interfere with hemolysis by causing a potentiating effect. It was not possible, however, to find cultures which produced useful quantities of δ-lysin free of α-lysin.

Materials and Methods

Cultures.—Cultures selected were those *S. aureus* isolates which produced the widest zones of hemolysis when streaked on agar plates containing washed human erythrocytes and incubated in an atmosphere of 25% carbon dioxide in oxygen. From these four cultures were selected (L.H. Nos. 1363, 2426, 2428, and 2429), which produced the highest yields of δ-lysin and which failed to cause hemolysis when grown anaerobically on sheep cell plates. The latter step was introduced to eliminate cultures producing β-lysin which, according to Christie and North (1) and confirmed by Elek and Levy (5) and Marks and Vaughan (13), is the only lysin produced by *S. aureus* under anaerobic conditions. The selected cultures produced both α- and δ-lysins.

Medium.—The medium described by Elek and Levy (4) modified by additional 2% proteose peptone (Difco No. 3) was used throughout.

Production of lysin.—For lysin production cultures were grown on cellophane sheets laid over solid medium as described by Marks and Vaughan (13). With the exception that plates were incubated for 22–24 hours, the procedure of Marks and Vaughan (13) was followed. The lysin was recovered from the plates as described by the above authors and the cells removed from the liquid immediately after harvesting by centrifuging at 12,000–14,000 r.p.m. and at 0° C. The clear supernatant was poured from the centrifuge tubes and stored at −20° C.

Partial purification.—Unless otherwise stated the lysin employed was partially purified in the following manner. The crude solution was cooled to 0° C, adjusted to pH 4.0 by addition of 0.1 *N* acetic acid. Cold methanol (−20° C) was added to a final concentration of 25% (v/v) to precipitate the lysin. The precipitate was collected by centrifuging at −5° C and dissolved in cold saline–phosphate buffer (8) at pH 6.8, dialyzed for 3–4 hours against three changes of the same buffer, and then stored at −20° C. Details of this procedure will be published elsewhere (10). During the course of these studies it was found (10) that the mixed lysins, either crude or once precipitated, could be freed of α-lysin by heating at 60° C for 10 minutes. Some experiments were conducted with δ-lysin freed of α-lysin in this manner.

Determination of hemolytic activity.—One milliliter of a 7% suspension of washed erythrocytes in saline–phosphate buffer or physiological saline is added to a mixture of 1 ml lysin suitably diluted in the above buffer together with 5 ml buffer or the indicated volume of other solution with buffer to give a total volume of 7 ml. After the desired period of incubation, the unhemolyzed cells are removed by centrifuging, the supernatant solution diluted 1 ml to 6 ml of 0.1% sodium carbonate, and its optical density determined at 541 mμ. A 1% suspension of human erythrocytes was found to contain approximately 1.1×10^8 cells per ml and when lysed with sodium carbonate solution (1 ml suspension plus 6 ml carbonate solution) has an optical density of 0.360 at 541 mμ. A Coleman Jr. spectrophotometer and 18-mm diameter curvettes were used throughout. Buffers were prepared by combining 0.028 *M* disodium phosphate and 0.084 *M* monopotassium phosphate solutions to give the desired pH and mixing these buffers with an equal volume of 0.286 *N* sodium chloride solution (total ionic strength 0.183). Details of the above procedures and reagents are given elsewhere (8). Erythrocytes of human origin were employed unless otherwise stated. The extent of hemolysis is reported as the optical density of the hemoglobin released when diluted as described above.

Results

Effect of α-Lysin on Hemolysis of Human Erythrocytes by δ-Lysin

Since early attempts to prepare δ-lysin free of α-lysin had been unsuccessful it was necessary to re-examine the effect of α-lysin on hemolysis of human erythrocytes by δ-lysin.

This effect was determined by comparing the hemolytic activity against human erythrocytes of the mixed lysins and a sample in which the α-lysin had been neutralized by addition of α-antitoxin. The data presented in Table I show that the addition of sufficient α-antitoxin to neutralize the α-lysin present in the mixture, as indicated by the rabbit erythrocytes, caused

TABLE I

EFFECT OF α–LYSIN ON HEMOLYSIS OF HUMAN AND RABBIT ERYTHROCYTES BY CRUDE δ-LYSIN

α-Antitoxin, units/ml	Hemolysis*	
	Human cells	Rabbit cells
Nil	0.169	0.280
0.125	0.170	0.070
0.250	0.171	0.072
0.50	0.168	0.082
1.0	0.169	0.072
20	0.170	0.078
Complete hemolysis	0.285	0.290

*Concentration of hemoglobin released reported as optical density (D_{541}) after dilution of supernatant, 1 ml in 6 ml of 0.1% sodium carbonate.

no detectable change in the capacity of δ-lysin to hemolyze human erythrocytes. It may be concluded, therefore, that α-lysin does not act synergistically with δ-lysin during lysis of these cells. Thus hemolysis by a preparation containing both α- and δ-lysins can be attributed to δ-lysin alone and the presence of α-lysin does not interfere.

Hemolysis at Various pH Levels

The following experiments were conducted using saline–phosphate buffers at isoionic strength prepared as previously described (7). The results obtained are shown in Fig. 1. It should be noted that α-antitoxin at a concentration found adequate to neutralize the α-lysin present was employed in the experiments with rabbit erythrocytes. It will be seen that activity of the lysin is at a minimum in the range of pH 6.8–7.3. The results obtained are similar regardless of the type of erythrocytes used. Although not shown here, preparations heated at 60° C for 10 minutes to inactivate the α-lysin gave results similar to those shown in Fig. 1. Owing to the fact that it was not possible to discover a pH at which this lysin exhibits maximum activity, and owing to the rather marked effect on activity of slight changes in pH at points removed from pH 6.8–7.0 it was decided to conduct tests at pH 6.8 where slight deviations in hydrogen ion concentration would have minimum effects on the rate of hemolysis. In addition, buffers of this pH may be employed with either α- or δ-lysins (8).

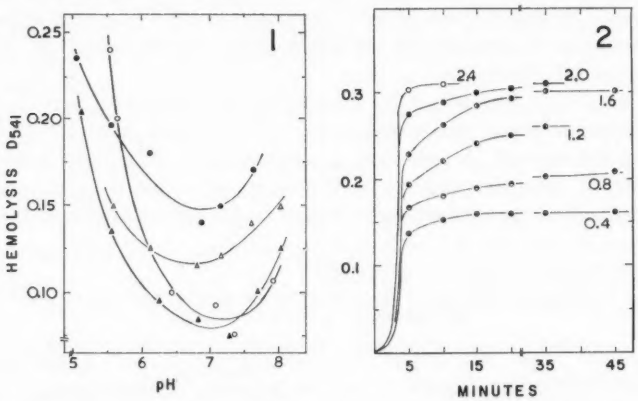

FIG. 1. Effect of pH on hemolysis by δ-lysin from four different cultures: ○, 1363; ●, 2426; ▲, 2428; △, 2429.

FIG. 2. Time–hemolysis relationships with different lysin concentrations. Quantities in milliliters of diluted lysin as indicated. Lysin from culture No. 2428 employed.

Lysin Concentration vs. Time

Aliquots of human erythrocytes prepared in the usual fashion were added to a series of different concentrations of lysin and incubated at 37° C. Samples were removed after the periods of incubation shown in Fig. 2 and centrifuged immediately to remove unhemolyzed cells. The characteristic pattern with

all levels of lysin employed was one of rapid hemolysis followed by an abrupt cessation or a very slow rate of lysis. Similar results (Fig. 3, curves 1 and 3) were obtained with δ-lysin preparations freed of α-lysin activity by heating.

As the possibility existed that the results shown in Fig. 2 were characteristic of human erythrocytes rather than the lysin, the experiment was repeated using rabbit erythrocytes and sufficient α-antitoxin to neutralize the α-lysin present in the δ-lysin preparation. It was found, however (Fig. 3, curves 2 and 4), that substitution of rabbit for human erythrocytes did not materially alter the shape of the curves obtained. Hence it may be concluded that the time–hemolysis curves shown in Figs. 2 and 3 are characteristic of those to be obtained with δ-lysin. These observations suggest that the relationship between δ-lysin concentration and extent of hemolysis may be examined employing relatively short incubation periods.

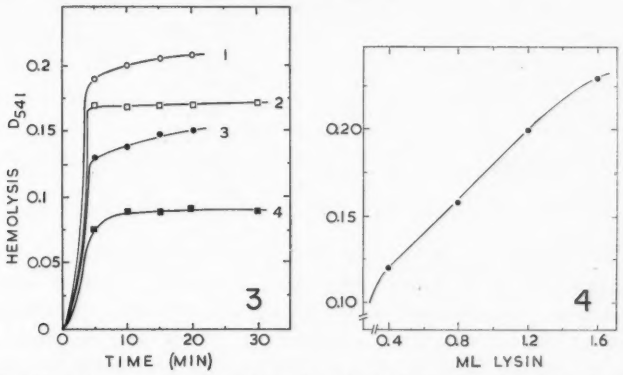

FIG. 3. Time–hemolysis relationships; Nos. 1 and 3, heat-treated lysin, 2 and 0.4 ml respectively, with human erythrocytes; Nos. 2 and 4, crude lysin (α + δ) + α-antitoxin to neutralize α-lysin, 2.8 and 1.6 ml toxin respectively, with rabbit erythrocytes. Lysin from culture No. 2428 employed.

FIG. 4. Relationship of δ-lysin concentration to hemolysis.

Relationship of Lysin Concentration to Hemolysis

If a series of concentrations of δ-lysin are employed with a constant incubation period of 10 minutes and the extent of resulting hemolysis is plotted against lysin concentration the curve shown in Fig. 4 is obtained. It will be observed that the quantity of hemoglobin released within the optical density range from 0.120–0.235 (30–65% hemolysis) may be related linearly to the lysin concentration employed. This range of hemolysis is covered by a fourfold increase in lysin concentration.

In all later experiments a constant incubation period of 10 minutes was employed.

Effect of Erythrocyte Concentration on Hemolysis

Another variable which may effect extent of hemolysis is the concentration of erythrocytes employed. Human erythrocytes prepared as usual were added at the concentrations indicated. The results of these experiments

(Table II) show that the proportion of cells hemolyzed is reduced as the concentration of red cells is increased. Hence, erythrocyte concentration must be controlled rather closely to reduce error from this source.

TABLE II

EFFECT OF VARYING ERYTHROCYTE CONCENTRATION ON HEMOLYSIS

Cell concn., %	Total Hb, D_{541}	Hb released, D_{541}	Hemolysis, %
0.172	0.062	0.057 0.057	91.9
0.52	0.186	0.156 0.153	83.2
1.30	0.470	0.292 0.292	62.2
1.78	0.640	0.370 0.370	57.8
2.39	0.860	0.432 0.440	50.7

Effect of Erythrocyte Constituents on Hemolysis by δ-Lysin

The abrupt change in rate of hemolysis by δ-lysin from an initial rapid one to a slower rate suggested that the products of the reaction may inhibit further lysis. Consequently, the effects of ghosts and hemoglobin upon hemolysis by δ-lysin were investigated. Human erythrocytes prepared as described under "Methods" were lysed by suspension in distilled water and the ghosts recovered and washed a number of times, alternatively, in distilled water and saline. They were finally suspended in saline–phosphate buffer (pH 6.8) at a concentration such that 1 ml contained the ghosts from 1 ml of a 7% erythrocyte suspension. The hemoglobin obtained from the water-lysed erythrocytes was diluted to give a concentration equivalent to approximately 90% hemolysis of a 7% suspension of cells. The effect of erythrocytes, ghosts, and hemoglobin are shown in Table III where it will be seen that both products of hemolysis have an inhibitory effect on hemolysis by δ-lysin. As the two products were added in approximately the same proportion as found in erythrocytes, it may be concluded that ghosts are considerably more inhibitory than the cell contents.

The Effect of Proteins on Hemolysis by δ-Lysin

Although Marks and Vaughan (13) reported that hemolysis by δ-lysin is inhibited by normal sera from a number of animal species and that both the albumin and globulin fractions are inhibitory, the relative inhibitory effects of proteins have not been studied previously. The materials to be investigated were either dissolved or diluted in the usual saline–phosphate buffer

and then added to the test system to give the final concentrations shown in Table IV. Lysin and proteins were mixed 15 minutes prior to addition of erythrocytes, whereas other test materials were added to the test system immediately before addition of erythrocytes. The results of these experiments indicated the general capacity of proteins to inhibit hemolysis by δ-lysin. Globulins appear to be the most inhibitory of the separate fractions studied, whereas both rabbit and human sera are much more inhibitory on a basis of protein content. It should be noted, however, that the whole globulin fraction, which is considered to contain all the antibodies, is less inhibitory than two globulin subfractions (IV*a*, IV*b*) which normally do not contain antibodies (2).

TABLE III

EFFECT OF ERYTHROCYTE CONSTITUENTS ON LYSIS BY δ-LYSIN

		Inhibitor	Hemolysis, D_{541}		Hemoglobin released	
δ-Lysin		–	0.183	0.170	0.183	0.170
No lysin	+	Ghosts	0.009	0.009		
δ-Lysin	+	Ghosts	0.078	0.082	0.069	0.073
No lysin	+	Hb	0.242	0.242		
δ-Lysin	+	Hb	0.358	0.362	0.116	0.120

NOTE: In tubes containing cell fractions, 1 ml of buffer was replaced with 1 ml of ghost suspension or hemoglobin solution prepared as described in text.

TABLE IV

EFFECT OF VARIOUS PROTEINS ON LYSIS BY δ-LYSIN

Protein	Final concentration	Hemolysis,* %
Control	–	57
Gelatin	1%	40
Albumin	1%	38
Gamma globulin	1%	3
Gamma globulin	0.1%	27
Fraction IV-1	0.064%	6
Fraction IV-4	0.072%	4
Human serum	1:3584	33
Rabbit serum	1:3584	29

*Average of duplicates.

Effect of Glucose, Citrate, and Magnesium Ions on Hemolysis

As it has been shown (8) that glucose and citrate inhibit hemolysis by α-lysin and magnesium ions activate β-lysin (11), the effect of these materials on hemolysis by δ-lysin was examined. Buffer solutions were prepared containing citrate or glucose at the concentrations found in Alsevers' solution (8) and adjusted by addition of sodium chloride to give a solution possessing

the same tonicity as the usual phosphate–saline buffer (8). Magnesium sulphate was added to the usual saline–phosphate buffer with care taken to maintain the desired pH by addition of *N* sodium hydroxide. The results of these experiments are shown in Table V. It is apparent that hemolysis by δ-lysin is unaffected by any of the added materials.

TABLE V

EFFECT OF VARIOUS ORGANIC COMPOUNDS AND OF MAGNESIUM ON HEMOLYSIS BY δ-LYSIN

Materials added	Hemolysis, D_{541}	
Control (pH 6.1)	0.202	0.212
Citrate (5.7 mg/ml)*	0.210	0.203
Glucose (14 mg/ml)†	0.210	0.200
Alsevers' solution‡	0.211	0.219
$MgSO_4$ (1.43×10^{-3} *M*)+	0.355	0.360
$MgSO_4$ (1.43×10^{-4} *M*)+	0.375	0.370
Control§	0.380	0.375

*Buffer solution contains 0.91 g trisodium citrate.$2H_2O$; 0.585 g sodium chloride dissolved in 45 ml phosphate buffer, pH 6.1, ionic strength 0.08, diluted to 100 ml with water. Five milliliters replaced 5 ml of usual buffer.

†Solution contains 2.05 g glucose replacing citrate, otherwise as above.

‡Alsevers' solution contained 2.05 g glucose, 0.8 g sodium citrate, 0.42 g sodium chloride per 100 ml (pH 6.1). Obtained from Baxter Laboratories of Canada Ltd., Acton, Ontario.

§In usual saline–phosphate buffer, pH 6.8.

Summary of Method for the Determination of the Hemolytic Activity of δ-Lysin

A method of determining δ-lysin activity is proposed, based on the capacity of this lysin to lyse human erythrocytes. Conditions found suitable for this determination by a partial hemolysis procedure have been described above. They may be summarized as follows:

The test is conducted employing human erythrocytes and diluted lysin, all in saline–phosphate buffer, pH 6.8, and a final concentration of 1% erythrocytes.

Hemolysis is allowed to proceed for 10 minutes. Centrifugation to remove unhemolyzed erythrocytes is begun at precisely 10 minutes after addition of the erythrocyte suspension to the diluted lysin (8).

Determination of the optical density of the released hemoglobin and relationship of degree of hemolysis to lysin concentration have been described.

The method may be employed to determine the relative activity of a number of lysin preparations, using a single lot of erythrocytes. Experience with this procedure over a period of 18 months employing numerous different lots of blood indicates that the end result is greatly influenced by the lot and age of blood employed. In the absense of a lysin reference standard these factors preclude use of the method for accurate comparison of the activity of different samples of lysin determined at different times. A closer examination of the lysin is necessary, however, before such a standard may be established.

Discussion

In addition to the determination of the conditions under which a satisfactory assay of δ-lysin may be made the present study revealed some characteristics of this lysin hitherto unreported. The most significant of these is the shape of the time–hemolysis curve obtained with the lysin. The characteristic curve is one indicating an initial rapid rate of hemolysis followed by an abrupt change to a slower rate. In this respect δ-lysin is quite different from α-lysin (8) and a number of other bacterial lysins (3) which typically give sigmoid time–hemolysis curves. The curves obtained for δ-lysin in the present studies resemble those obtained by Bernheimer (3) employing tyrocidin. In the latter instance, the change from a rapid to a slower rate of hemolysis was found (3) to be due to the accumulation of erythrocyte ghosts, whereas the change in rate observed with δ-lysin appears to be associated with the accumulation of ghosts and hemoglobin in the test system, as erythrocytes are lysed by the lysin.

A second difference between α- and δ-lysins was observed when it was found that hemolysis by δ-lysin is unaffected by the presence of glucose or citrate, whereas both inhibit hemolysis by α-lysin (8). Hemolysis by δ-lysin is likewise unaffected by magnesium ions while this ion causes a marked activation of β-lysin as a hemolytic agent (11).

The conclusion by Marks and Vaughan (13) that the presence of α-lysin in δ-lysin preparations does not interfere with the determination of hemolytic activity of δ-lysin when erythrocytes of human origin are employed is confirmed by the observation that neutralization of α-lysin with α-antitoxin added to a mixture of α-and δ-lysins does not reduce the capacity of the mixture to hemolyze human erythrocytes. Although this result may have been expected from previous observations (13), to the effect that α-lysin does not cause lysis of human erythrocytes, it also rules out the possibility that it may act synergistically with δ-lysin.

The general capacity of proteins to inhibit the hemolytic activity of δ-lysin is demonstrated and confirms the observation of Marks and Vaughan (13) that both the globulin and the albumin fractions of serum and whole sera are inhibitory in this system. Furthermore on a basis of nitrogen content, globulin subfractions (IV*a* and IV*b*) considered to be normally free from antibodies (2) are more effective than whole globulin as inhibitors of hemolysis. Although these observations may suggest that δ-lysin should have little effect in vivo, nevertheless injection of the lysin into the skin of experimental animals has been reported (13) to produce a dermonecrotic reaction.

The effect of variation in pH on hemolysis by δ-lysin is quite different from the effect of this variable upon hemolysis by α-lysin. Hemolysis by δ-lysin is minimal at pH 6.8–7.0 with increasing rates of lysis on both the acid and alkaline sides of neutrality, whereas α-lysin shows a marked optimum at pH 6.8–7.0. The curves obtained in the present experiments with δ-lysin resemble those reported by Rud (15) employing a crude preparation designated by the author as "staphylolysin".

The proposed method of determining δ-lysin activity based on its hemolytic capacity is particularly useful in view of the absence of information regarding its antigenic properties and of the rather general inhibitory effects of protein which may reduce the value of a classical antigen–antibody titration as a means of determining the δ-lysin content of a preparation.

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A SECONDARY EFFECT OF DDT UPON THE AQUATIC MICROFLORA AND MICROFAUNA[1]

MARSHALL LAIRD[2]

Abstract

Observations were made on the microflora and microfauna of infusions in which larvae of *Anopheles maculipennis atroparvus* were being subjected to DDT selection pressure (2 μg/ml). Comparisons with controls indicated that the insecticide influenced neither the species representation nor the succession of the organisms concerned. However, heavy initial larval mortality took place. Dead larvae remaining in the containers overnight became centers of very rapid bacterial multiplication, and the maturation of the experimental infusions was accelerated. The larval cultures became polysaprobic after about two weeks, by which time the surviving larvae were in the fourth instar. These survivors were large and healthy, having spent their early developmental period in the presence of a rich supply of infusion organisms answering their food requirements, and successfully withstood the deteriorating environmental conditions and consequent heavy infestation with epibionts (Chlamydobacteriales and peritrichous ciliates). On the other hand, fourth instar larvae from similar infusions in the laboratory colony of *A. m. atroparvus* were often undersized and sickly because of early overcrowding linked with the initially longer time required for the development of an adequate yield of food organisms. Such larvae, also pupae, frequently died of exhaustion due to undernourishment and complicated by the hampering of their activity by a vestiture of bacteria and ciliates. This explained the paradox that adults derived from the laboratory colony tended to be less robust than those hatching from developmental stages subjected to DDT selection pressure. Besides pointing to the importance of paying due regard to larval environmental factors in resistance studies, these observations suggest the need for field investigations of the significance of similar chains of events in nature.

Introduction

I spent the months of September and October, 1956, at the laboratory of Professor Guido Frizzi in the Lazzaro Spallanzani Institute of Zoology, Pavia. Research in progress there included studies of *Anopheles* (*Anopheles*) *maculipennis atroparvus* van Thiel subjected to DDT selection pressure by the rearing of larvae in tap water with 2 μg/ml of the insecticide. It had been noted that imagines derived from such larvae were frequently more robust than those bred in the standard laboratory colony. Furthermore, fatally terminating vorticellid infestations had already been recorded from the latter (14). These facts suggested that it would be worth while to investigate the microflora and microfauna of the experimental and colony larval cultures, to determine whether the former were affected by DDT. The literature has little to offer on this subject, beyond such statements as those that various members of the zooplankton seem to be more resistant to DDT than aquatic

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[2]Institute of Parasitology, McGill University, Macdonald College.

insects (7), and that the members of numerous phyla, including the Protozoa, Nemathelminthes, Platyhelminthes, and Rotatoria, are resistant to contact poisoning with DDT because they lack a chitinous cuticle (3).

Methods

Professor Frizzi's data on his DDT selection pressure experiments are not yet published, and therefore no details of his methods shall be given beyond the essential facts that he allowed batches of *A. m. atroparvus* eggs to hatch in white enamelled basins containing unboiled tap water with 2 μg/ml of DDT, and that wheat bran and yeast were added to provide suitable conditions for the growth of infusion organisms to serve as food for the developing larvae. Temperature and other factors are responsible for considerable variation in the duration of the aquatic cycle of this mosquito, approximately three weeks being required at a temperature of 23° C. (4). Insectary temperatures during the investigation fluctuated about this level. Experiments were thus conducted for 21 days, eight cultures, four with DDT and larvae and four without either, being studied. Daily surface and bottom samples were pipetted from each container, and three drops from each of these samples were placed on microscope slides and covered with 22 mm. sq. cover slips (No. 1). The preparations were then searched microscopically, using phase-contrast equipment In addition, material concentrated by centrifuging was collected on albumen-treated cover slips, fixed in Schaudinn's fluid, and stained with Heidenhain's iron haematoxylin. Tentative identifications based upon fresh material were confirmed or corrected from the permanent preparations.

Data and Discussion

The following organisms were identified, all of them being recorded from both the DDT infusions and their controls (and subsequently from the laboratory colony of *A. m. atroparvus* as well).

BACTERIA:* *Spirillum undula* (Müller), *S. volutans* Ehrenberg, *Sphaerotilus natans* Kützing, *Zoogloea ramigera* Itzigsohn, *Beggiatoa alba* (Vaucher), *Spirochaeta plicatilis* Ehrenberg, and undetermined micrococci, bacilli, and spore-bearing rods.

PROTOZOA: (**Mastigophora**) *Cryptomonas erosa* Ehrenberg, *Chilomonas paramecium* Ehrenberg, *Chlamydomonas ehrenbergi* Goroschankin, *Peranema granulifera* Penard, *Oikomonas termo* (Ehrenberg), *Monas* sp., *Bodo caudatus* (Dujardin), *Bodo minimus* Klebs, *Bodo* spp., *Pleuromonas jaculans* Perty, *Tetramitus pyriformis* Klebs. (**Sarcodina**) *Amoeba proteus* (Pallas), *Vahlkampfia limax* (Dujardin). (**Ciliata**) *Enchelys* sp., *Chilodonella uncinata*

*Identifications of bacteria, like those of the other organisms listed, are based upon morphological criteria alone. Thus specific identifications were only possible in the case of a few relatively large and distinctive bacteria. The size and form of these species were as described in the sixth edition of Bergey's *Manual of Determinative Bacteriology* (2) and Liebmann's *Handbook of the Biology of Fresh and Polluted Water* (13). Unfortunately, the circumstances of the investigation did not permit the employment of bacteriological culture techniques, and the smaller bacteria, which constituted the bulk of the flora and were obviously of considerable ecological significance, had to remain undetermined.

(Ehrenberg), *Paramecium caudatum* Ehrenberg, *Colpoda culcullus* Müller, *Colpidium colpoda* (Ehrenberg), *Saprophilus muscorum* Kahl, *Cyclidium citrullus* Cohn, *Epalxis exigua* Penard, *Oxytricha fallax* Stein, *Pyxidium invaginatum* Stokes, *Vorticella convallaria* (L.), *V. microstoma* Ehrenberg. (**Suctoria**) *Podophrya fixa* Müller.

ROTATORIA: *Rotaria neptunia* (Ehrenberg).

All these plants and animals are well known from infusions and from stagnant waters generally, and many of them are in common use as biological indicators of pollution. Their descriptions appear in standard texts (2, 11, 13).

The microscopically identifiable elements of the microflora and microfauna not only occurred in the infusions containing DDT as well as in the controls, but their relative order of appearance corresponded in both cases. Numerous investigators have studied the succession of protozoan populations in infusions, Woodruff (19) quoting references dating back to 1703. Kent (10) stated that various monads and other flagellates, and ciliates of the genera *Paramecium*, *Colpoda*, *Cyclidium*, *Oxytricha*, and *Vorticella* follow one another in hay infusions (actual order of succession not stated), in which Woodruff (19) established the following sequence:

1. Monads (*Bodo*, *Monas*, *Oikomonas*, and other small flagellates).
2. *Colpoda* (notably *C. cucullus* mixed with *Colpidium*).
3. Hypotricha (*Oxytricha*, *Stylonychia*, etc.)
4. *Paramecium* (*P. caudatum* and *P. aurelia*) (after deliberate seeding).
5. *Vorticella* (several spp.).
6. *Amoeba* (including *A. proteus*).

Eddy (6) failed to observe any strict regularity of appearance of Protozoa in infusions prepared with various plants, but noted that monads were plentiful in the early stages in practically all cases and that *Colpoda* and *Colpidium* were usually the first ciliates to appear. Unger (18), who also dealt with a number of different plant infusions, reported that *Bodo minimus* or *Bodo* sp. always appeared first, being closely followed by *Colpoda inflata*, *Colpoda* sp., or *Enchelys pupa*, and latterly by *Astasia* sp., *Bodo* spp., *Oikomonas termo*, *Amoeba* sp., and rotifers.

The organisms named below, which were common in all the Pavia cultures and are regarded as characteristic of the infusions studied, bloomed in the order in which they are listed:

1. Bacilli and micrococci, Bodonidae (*Bodo minimus*, *Bodo* sp., *Pleuromonas jaculans*).
2. *Vahlkampfia limax*, *Colpoda cucullus*, *Colpidium colpoda*, *Chilodonella uncinata*.
3. *Beggiatoa alba*, *Spirochaeta plicatilis*, *Cyclidium citrullus*, *Oxytricha fallax*, *Epalxis exigua*.
4. *Spirillum* spp., felted growths of Chlamydobacteriales (*Beggiatoa/Sphaerotilus/Zoogloea*), *Vorticella microstoma*, *Podophrya fixa*.
5. *Chilomonas paramecium*, *Amoeba proteus*.
6. *Rotaria neptunia*.

This over-all succession broadly agrees with the usual sequence of events marking stages in the transition from mesosaprobic to polysaprobic conditions (13). The appearance of *Vahlkampfia* and *Chilodonella* at so early a stage was perhaps due to the use of unsterilized tap water in making up the infusions, for Woodruff (19) conducted some experiments with unboiled tap water and found that monads, small amoebae, *Chilomonas*, and *Chilodonella* (= *Chilodon*) were present from the start. The same author, confirming earlier observations by Kent, recorded active *Colpoda*, *Chilodonella*, and small flagellates from moisture on blades of grass. *Paramecium caudatum* was numerous, together with *Cyclidium* and Hypotricha, in two infusions only. It is well known that this species is not a regular inhabitant of such cultures, and it is necessary to seed infusions, as Woodruff did, to ensure its appearance. No attempt was made to trace the origin of the spores and cysts from which these organisms originated. Some were doubtless present in the tap water, others on the bran added at the outset, and still others might have dropped onto the surface of the uncovered infusions during their maturation.

Several of the species recorded made their first appearance, in relatively small numbers, well before their actual blooming. Thus *Vahlkampfia limax* and *Chilodonella uncinata* were each once recorded on the first day, and *Vorticella microstoma*, a polysaprobic ciliate flourishing in aging infusions (after 10–14 days in the present investigations) was once noted as early as the second day. Again, some of the forms persisted in dense concentrations for some days after blooming. For example, *Colpoda cucullus* and *Cyclidium citrullus* usually remained abundant until stages 4 and 5 respectively; and the Bodonidae, which never entirely disappeared, flourished again in stages 5 and 6.

The only organisms primarily most abundant at the bottom and on the sides of the culture basins were the Chlamydobacteriales, *Amoeba proteus*, *Podophrya fixa*, and *Rotaria neptunia*. The remainder—even *Vorticella microstoma*, which sometimes became abundant attached to surface zoogloeal scum—thrived in the topmost zone of the medium, only sinking to the bottom in massive concentrations shortly before, to quote Woodruff (19), their "more or less rapid elimination as an important factor in the life of the infusion".

Time did not allow the study of the interrelated factors determining succession—food chains, reproductive potential, dissolved O_2 and CO_2, pH, temperature, and the rest. Attention was concentrated upon a single aspect of the findings, the fact that although the same microscopic plants and animals were recorded from the control and experimental basins alike, and similar sequences were observed in both cases, the rate of succession was initially more rapid in the infusions containing DDT.

The phenomenon proved to have a simple explanation. During the first 2 days there was considerable larval mortality due to the insecticide, and the bodies of larvae dying overnight became centers of intense bacterial activity. The acceleration of bacterial development in its turn stimulated protozoan growth and multiplication. *Bodo* spp. and *Pleuromonas jaculans* became as abundant by the end of the first day as they did in 3 days in the control infusions, and by the third day *Colpoda*, *Colpidium*, and *Chilodonella* were

thriving—it took 6 or 7 days for the control populations of these ciliates to attain similar levels. The third step towards maturation was completed by the experimental infusions after a total of 6 or 7 days had elapsed, this being about three days less than were required by the controls. A predominance of felted bacteria and stalked ciliates and suctorians marked the fourth stage in succession, which was reached in from 10 to 14 days. It became increasingly difficult to make arbitrary distinctions between the experimental and control microflora and microfauna between the third and fourth stages, and thereafter the infusions were in a state of biological balance with one another.

The consequences of the chain of events just outlined had an important bearing upon the development of *A. m. atroparvus* in the culture basins. The surviving experimental larvae had a rich food supply from the outset—although we still have much to learn concerning the nutrition of mosquito larvae, it may be accepted for all practical purposes that almost any abundant plankton organism, be it bacterial, algal, or protozoan, constitutes a sufficient source of food for larval anophelines (1). At the same time, though, it is generally recognized that laboratory infusions are liable to "turn bad" and cease to be suitable for these larvae. Rozeboom (15) stated that hay infusions could be regarded as suitable for the rearing of *A. albimanus* as long as ciliates of the genus *Paramecium* were present. These protozoans reach their peak of abundance under α-mesosaprobic conditions, and Rozeboom's assertion really reflects the fact that the transition from mesosaprobic to polysaprobic conditions can be withstood by relatively few species of mosquitoes. Although suitable food organisms continue to be present—Barber (1) was able to rear anophelines to maturity in pure cultures of *Spirillum* spp., which abound in polysaprobic waters—bacteria-induced changes in environmental chemistry, and the flourishing of epibiotic bacteria and protozoans (12) prejudice the chances of survival of larvae not adapted to such waters.

As north Italian *A. m. atroparvus* may occur in nature in mesosaprobic waters (8), questions of the possible unfavorability of the larval environment did not arise until the development of a typically polysaprobic microflora and microfauna. This took place at the climax of the fourth stage in the succession of the infusion organisms, after 10 to 14 days, and during the term of the fourth larval instar. Until then, dissections always revealed an abundance of ingested protozoans of fairly large size in the anterior part of the alimentary tract. Ciliates of the genera *Colpoda*, *Chilodonella*, and *Oxytricha* were always prominent. Bodonid flagellates and the extremely common *Cyclidium citrullus* were presumably ingested in numbers as well, but owing to their small size and relatively delicate pellicle it was difficult to identify them among the gut contents. At the peak of the fourth stage in succession, though, predation by *Podophrya fixa* reached such a level that populations of the surviving free-living ciliates, among which *Cyclidium citrullus* was still prominent, were severely depleted. The fourth instar larvae thus had to complete their development in a medium in which the commonest organisms were bacteria, small flagellates, attached vorticellids, and the primarily bottom-dwelling *Podophrya*

fixa and *Amoeba proteus*. In addition, they were subject to infestation with epibionts, particularly *Vorticella microstoma*, and to a lesser extent *Sphaerotilus natans* and *Pyxidium invaginatum*. Nevertheless, they invariably pupated; and the pupae, themselves often bearing numerous vorticellids, always gave rise to healthy adults.

In the main laboratory colony of *A. m. atroparvus*, although the rearing procedure was the same as that already described, larvae were often undersized and of sickly appearance. These, and the resultant pupae, became very heavily infested with *Vorticella microstoma*, and death sometimes took place as the time for hatching approached (14). It is submitted that this state of affairs may be explained on the basis of the course of events in the control infusions. Early larval mortality in the colony being negligible, the infusions failed to show the initial acceleration in the rate of succession that characterized the experiments with DDT. Vigorous competition thus took place for the initially poorer food supply, 3 days (instead of 1, in the case of the experimental cultures) being required for the blooming of *Bodo* spp. and *Pleuromonas jaculans*, 6 or 7 days (instead of 3, in the case of the experimental cultures) for that of *Colpoda*, *Colpidium*, and *Chilodonella*, and some 9 or 10 days (instead of 6 or 7) for that of *Cyclidium* and the Hypotricha. Competition for these organisms appeared to prevent their flourishing as freely as in the experimental and control infusions, and the dissection of second and third instar larvae revealed markedly fewer ingested protozoans than in the case of the survivors of the selection pressure experiments. In brief, the early picture was one of undernourishment due to overcrowding, never a factor in the development of the experimental larvae, reduced in numbers as they were by the DDT-induced initial mortality which brought about a rapid acceleration of infusion development. Overcrowding and undernourishment lead to the lengthening of the larval growth period and to a rise in mortality as larval growth proceeds (16). The second of these consequences, and perhaps the accumulation of larval faeces as well, helped to accelerate the later stages in the succession of the colony infusion organisms, with the result that the blooming of *Sphaerotilus* and *Vorticella* occurred within 10–14 days as in the experimental infusions. Because of their lengthened growth period some of the colony larvae were still in the third instar at this stage, while the remainder had only recently undergone ecdysis. These undernourished larvae thus had to spend all, or nearly all, of the comparatively protracted fourth instar phase, which involves not only the greatest mass increment but also the expenditure of much energy during profound tissue transformations (9), under relatively unfavorable polysaprobic conditions. At the same time, both the larvae and the resultant pupae became heavily infested with *Vorticella microstoma*, sometimes together with *Sphaerotilus natans*. Although the tissues of the developing mosquitoes were never actually invaded by *Sphaerotilus*, as may happen when extreme undernourishment leads to a weakening of the chitinous cuticle (12), many of these insects became so hampered by their vestiture of epibionts that it is hardly surprising that they died of exhaustion at or towards the crises of pupation or hatching.

These findings are considered a sufficient explanation for the mortality of vorticellid-infested *A. m. atroparvus* pupae in the Pavia laboratory colony, recorded by Micks (14). They also indicate why imagines derived from larvae subjected to DDT selection pressure in this laboratory often proved more robust than their controls. Adult viability and behavior are profoundly affected by larval nutrition, and Terzian and Stahler (17) have shown that *A. quadrimaculatus* derived from underpopulated colonies have a higher biting rate than those from overcrowded colonies, the biting rate in turn being related to the male/female ratio.

Crowell (5) experienced similar troubles due to undernourishment in the mass rearing of *A. quadrimaculatus* in pans containing hay or wheat infusion and yeast. Although a colony was established and maintained by this technique, it was found that because of the difficulty in maintaining a proper balance between the larvae and their food, the larvae exhibited considerable variation in size, the adults were smaller than wild-caught ones, and "larval, pupal, and adult mortalities were frequently high"—so high, in fact, that the extinction of the colony was threatened more than once. The problem was solved by daily supplemental feeding with pulverized dog biscuit, care being taken to regulate the amounts added so as not to leave a detrimental decomposing surplus.

It would be well to adopt this or an equivalent supplemental feeding procedure as a standard wherever a normal, healthy stock of a particular species of mosquito is required for experimental purposes. At the same time, attention is drawn to the possibility that even benign infestations with epibionts in the larval and pupal stages might have sufficient repercussions upon the resultant adults to influence the course of resistance studies. The answer to this does not merely lie in staving off the development of polysaprobic conditions, for although these favor the blooming of *Vorticella microstoma*, the ciliate first appears in relatively slightly polluted waters. It might not prove possible to eliminate epibionts short of developing bacteria-free nutrient media suitable for general use. Such media have already been devised and successfully used by individual workers, but they would have to be improved and standardized before their large-scale employment would be practicable. In the meantime, careful daily cleaning of the rearing pans, frequent replacement of the water with a clean, fresh supply, and possibly provision for aeration, are obvious partial remedies.

Quantitative investigation of several of the points touched upon in this brief study would be well worth while. For example, there is evidence that successive populations of infusion protozoans are each less numerous than their precursor (19), and this might prove to be a factor in the problems of larval nutrition raised herein. It would certainly be of decided interest to examine the effects of insecticide-induced acceleration of the microfloral and microfaunal succession in natural waters in which resistant mosquito larvae were developing in the presence of the decomposing remains of large numbers of non-resistant arthropods.

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STAPHYLOCOCCAL TOXINS

III. PARTIAL PURIFICATION AND SOME PROPERTIES OF δ-LYSIN[1]

A. W. JACKSON AND R. M. LITTLE

Abstract

A method is described by which δ-lysin may be separated from α-lysin and enzymes present in crude preparations. The procedure consists of precipitation of lysin from crude extracts under controlled conditions of ethanol concentration, temperature, and pH, followed by extraction of δ-lysin from the precipitate with 75% ethanol in saline–phosphate buffer, pH 6.8. Evidence is presented which indicates that crude δ-lysin consists of two fractions: one stable to heating at 60° C and another rapidly inactivated under these conditions. Heat-stable δ-lysin may be freed of contaminating α-lysin by heating crude preparation at 60° C for 15 minutes. δ-Lysin is retained by ultrafine sintered-glass filters which permit passage of α-lysin.

Owing to the reported (3, 8) quite general association of α- with δ-lysin among the products of *Staphylococcus aureus* a method for the preparation of δ-lysin free of α-lysin would appear to be a first requirement if the effects of δ-lysin on animals are to be critically examined.

To date the only previously reported attempt to purify δ-lysin was made by Marks and Vaughan (9), who claim to have achieved partial purification of this material. Selected cultures were grown on cellophane laid over solid medium, and the δ-lysin was then extracted from the surface growth with alcohol. Average recovery of lysin by this method was approximately 25% of the total produced, but recoveries varied widely and unaccountably. Although the extract was reported to be free of α-lysin no evidence was presented in proof of this claim. Freedom from β-lysin was assured, however, by use of cultures known to be non-β-lysin producers.

Attempts by Marks and Vaughan (9) to purify δ-lysin by fractional precipitation with acetone were unsuccessful. It was found, however, that most of the α-lysin present in their δ-lysin preparations could be inactivated by heating at 65° C for 5 minutes, but heating at this temperature for 30 minutes would cause up to 75% loss of δ-lysin activity. The question of whether α-lysin may be completely inactivated by heating under conditions which would retain useful quantities of δ-lysin was not resolved. Gladstone and van Heyningen (5) have recently reported that δ-lysin is stable to autoclaving at 115° C for 20 minutes.

Another possibility of separating α- from δ-lysin was indicated by Marks and Vaughan (9), who found that the latter lysin is absorbed on seitz pads. Recovery of lysin from the pads was poor, however, which reduces the possible value of this method.

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The present studies were directed toward separation of δ- from α-lysin and enzymes known to be produced by *Staphylococcus aureus*, and hence, to provide a method by which δ-lysin may be prepared free of the other known exocellular products of this organism. During the course of the investigation a number of approaches were made to the problem of purification of δ-lysin and those experiments which show something of the nature of the lysin are included, with a description of a simple method for its partial purification.

Materials and Methods

The cultures, medium, and methods of producing δ-lysin and determining its activity are described elsewhere (6, 9).

Determination of nitrogen.—The nitrogen content of various fractions was determined by nesslerization of samples digested by the method of Koch and McMeakin (7).

Coagulase.—The coagulase content of preparations was determined by addition of 0.4 ml of lysin solution to 0.6 ml of a 1:6 dilution of citrated rabbit plasma, and the time required to clot at 37° C observed.

Preparation of hyaluronic acid.—This material was isolated from defatted pig skin by the method of Meyer and Chaffee (11), slightly modified. Mechanically defatted pig skin was minced and extracted with acetone according to the method of Schiller *et al.* (15) (4 lb acetone per 2500 g minced skin). The acetone-extracted skin was then suspended in 7 liters *N* sodium hydroxide solution and the procedure of Meyer and Chaffee (11) followed for isolation of hyaluronic acid with the exception that treatment of the extract with Lloyds reagent was omitted.

Hyaluronidase.—Hyaluronidase activity was determined by measuring the quantity of reducing sugar liberated by the action of this enzyme upon hyaluronic acid (13). Reducing sugars were determined colorimetrically (12).

Phosphatase.—Presence of this enzyme was detected by the liberation of free phenolphthalein from phenolphthalein–phosphate according to the method of Barber, Brooksbank, and Kuper (1).

Lipase.—Egg-yolk saline was prepared according to the method of McGaughey and Chu (10), and, after filtration, adjusted to pH 7.2. This extract was diluted immediately before use with 0.2% saline to give 1.2% (v/v) egg yolk. One-milliliter aliquots were placed in 12×75 mm test tubes, warmed to 37° C in a water bath, and 0.3 ml of the material to be assayed mixed with the egg-yolk extract. The time required to produce clouding was taken as an index of lipase activity (4). Material which failed to become clouded within 2 hours was considered free of lipase.

Protease.—Protease activity was determined by observing the clearing of heated plasma plates by crude or purified lysin. Plates were prepared according to the method of Christie and Wilson (2) employing a mixture of heated rabbit serum and nutrient agar poured in thin layers. The materials to be tested were added in solution to filter paper disks or penicillin-assay

cylinders placed on the surface of the poured and solidified plates, which were observed at intervals of one-half hour for evidence of clearing while being incubated at 37° C.

Results

Heat Inactivation of δ-Lysin

Since it is known (14) that α-lysin is inactivated by heating at 60° C for short periods of time and it has also been reported (9) that at least a portion of δ-lysin activity remains after similar treatment there appeared to be a possibility of selectively inactivating α-lysin by heating a mixture containing the two lysins. The following experiments were conducted to determine optimum conditions for destruction of α-lysin and retention of δ-lysin during heating. Crude preparations from three strains known to produce both lysins were heated at 60° C in a water bath, and aliquots were removed for testing after the periods of heating indicated in Table I. Parallel determinations were made employing rabbit erythrocytes with heated lysin alone and with adequate α-antitoxin to neutralize the α-lysin originally present. The results show that heating for 40 minutes removes all lysin neutralized by α-antitoxin, and that prolonging the treatment for a total of 2 hours causes no further reduction in activity. Furthermore, an examination of the

TABLE I

HEAT INACTIVATION OF α- AND δ-LYSINS AT 60° C

Culture No.	Period of heating (min)*			
	0	40	60	120
1363	0.330	0.030	0.030	0.038
+ α-antitoxin	0.050	0.038	0.034	0.038
2426	0.049	0.033	0.032	0.032
+ α-antitoxin	0.035	0.033	0.033	0.035
2428	0.350	0.163	0.160	0.159
+ α-antitoxin	0.200	0.160	0.159	0.162

*Average hemolysis of rabbit erythrocytes expressed as optical density (D_{541}) of hemoglobin released. Total hemolysis, $D_{541} - 0.360$.

samples to which α-antitoxin was added shows that some active material not neutralized by the antitoxin is lost during heating and that this varies depending upon the culture employed. Isolate number 2426 showed little loss of non-α-lysin activity upon heating, whereas the remaining two isolates showed relatively greater loss of this activity.

A more detailed examination of the time factor was undertaken; typical results are summarized in Table II. Two rather significant conclusions may be drawn from the data obtained. Firstly, α-lysin appears to be quantitatively inactivated by heating at 60° C for a period of 5 minutes. The addition of adequate α-antitoxin to neutralize the α-lysin originally present causes no reduction in activity when the heated sample is tested against rabbit cells.

TABLE II

EFFECT OF HEATING CRUDE LYSIN (CULTURE No. 2428) AT 60° C

Period heated 60° C, min	Hemolysis (D_{541}) of erythrocytes		
	Human	Rabbit	R. + α-antitoxin*
0	0.278	0.310	0.163
5	0.192	0.140	0.140
10	0.194	—	—
120	0.190	—	—

*α-Antitoxin, sufficient to neutralize α-lysin originally present, added prior to activity test.

Therefore δ-lysin preparations can be freed of α-lysin by heating at 60° C for short periods of time. In practice, however, heating is usually continued for 10–15 minutes. Secondly, there is a sharp drop in δ-lysin activity within a period of 5 minutes at 60° C with no significant change in activity when the heating is prolonged for 2 hours. A certain proportion of the δ-lysin remains active during this treatment. Presence in crude δ-lysin of heat-labile and heat-stable materials possessing δ-lysin activity is indicated, therefore, by the results presented above.

Extraction of δ-Lysin from Freeze-dried Crude Mixtures

Although δ-lysin may be freed of contaminating α-lysin by the procedure described above, this does not provide a means of concentrating it. Consequently, freeze-drying followed by dissolution of the product in a smaller volume of water was employed as a method of concentrating the lysin. Although it was found that all the activity originally present could be recovered by this method, the degree of concentration permissible was limited, however, by the quantity of solids present. Consequently an attempt was made to separate the δ-lysin from impurities by extraction of freeze-dried material with alcohol–water mixtures. Results of preliminary experiments indicated that extraction with 95% ethanol in water mixtures gave low recoveries of lysin. Typical results of extracting δ-lysin from freeze-dried crude lysin with 75% ethanol in water are shown in Table III. Dialyzed crude lysin was freeze-dried and extracted twice at 0° C with ethanol–water mixture,

TABLE III

EXTRACTION OF δ-LYSIN FROM FREEZE-DRIED CRUDE PREPARATIONS

Fraction	Activity,* units/ml	Recovery, %
1. Crude dialyzed	132	100
2. No. 1 (heated)	92	70
3. Alcohol ext. of dried lysin	83	63
4. No. 3 (heated)	83	63
5. Residue after extraction	1 (approx.)	—

*Average of duplicates.

first with a volume equivalent to two thirds of the original volume of dialyzed lysin employed and the insoluble residue then re-extracted with a volume of mixture equivalent to one half of the first. During extraction the solids were broken with a stirring rod into a fine suspension and then removed by centrifuging. The combined extracts were freed of the greater part of the alcohol present by distillation at 25° to 30° C under reduced pressure, but were not taken to dryness. The residues were then dissolved in saline–phosphate buffer at pH 6.8 and dialyzed against three lots of distilled water.

It will be observed (Table III) that a large portion of the δ-lysin was extracted from freeze-dried crude lysin with the ethanol–water mixture, and that the portion thus recovered was stable to heating at 60° C for 10 minutes. All but a trace of δ-lysin was removed from the insoluble residue, hence the heat-labile portion of the original lysin is lost at some stage in the procedure.

Precipitation of δ-Lysin and Purification by Extraction

An alternative method of concentrating proteins is through precipitation, and as this method may have some advantages over freeze-drying it was examined as a means of preparing materials for the extraction procedure described above.

Active material was precipitated from crude lysin with ethanol at low temperatures (16) as follows: The crude lysin in physiological saline was first cooled to 0° C, then adjusted to the indicated pH by careful addition of cold *M* acetate buffer, pH 3.0. Ethanol, previously cooled to −20° C or lower, was then slowly added to the mixtures, with stirring, to give a final concentration of 25% ethanol (v/v). The temperature of the mixture was reduced to −5° C during addition of ethanol. After the mixture had been allowed to stand for at least 5 minutes, the precipitate was spun down at −5° C in a refrigerated centrifuge, the supernatant discarded, and the precipitate dissolved in chilled saline–phosphate buffer at pH 6.8. Recovery of δ-lysin by precipitation at different pH levels is shown in Table IV. It will be observed that variations in the pH at which precipitation occurs

TABLE IV

PRECIPITATION AND EXTRACTION OF δ-LYSIN

Treatment	Activity recovered,* %	Activity, units/mg *N*	Loss activity with α-antitoxin,† %
1. Original (crude)	100	434	40
2. Precipitated (pH 4.0)	98	1946	40
3. Precipitated (pH 4.3)	73	—	48
4. Precipitated (pH 4.95)	57	—	74
5. No. 2 ext'd with ETOH + buffer (pH 6.8)	38	3200	5
6. No. 5 heated 60° C, 15 min	No detectable loss		—

*Average of duplicates.
†Average of duplicates, employing rabbit erythrocytes.

have a marked effect on recovery of lysin, with close to complete recovery at pH 4.0 and less than 60% at pH 4.95. In addition, δ-lysin precipitated at the lower pH value contains relatively less α-lysin than that recovered at the higher value as indicated by the loss of activity upon addition of α-antitoxin. Hence, precipitation of δ-lysin at pH 4.0 with ethanol gives close to complete recovery of lysin, improves activity in terms of nitrogen content, but does not eliminate α-lysin.

δ-Lysin may be extracted from the precipitate by the technique described in the preceding section. Material precipitated as described above was extracted with 75% ethanol made up by adding 22 ml of saline–phosphate buffer pH 6.8, to 78 ml of 95% ethanol. Extractions may be done at 10° to 15° C without destruction of the lysin. Approximately 40% of the original lysin is recovered, but the material obtained is stable to heating at 60° C for 10 minutes, and therefore, like the lysin extracted in a similar manner from the freeze-dried material, does not contain the heat-labile fraction found in the crude preparation. Lysin prepared in this manner contains not more than a trace of α-lysin as neither the heat treatment nor addition of α-antitoxin causes loss of activity against rabbit erythrocytes.

Crude lysin employed for these studies contains phosphatase, hyaluronidase, and lipase but no detectable coagulase, fibrinolysin, or proteinase. Failure of the culture to produce coagulase upon the medium employed does not indicate that the organism is coagulase-negative since growth on suitable medium resulted in coagulase production.

It will be seen (Table V) that the enzymes present in the crude lysin were precipitated almost quantitatively by the procedure employed to recover the lysins. δ-Lysin, purified by extraction, contained no detectable lipase, phosphatase, or hyaluronidase and hence the procedure does separate this lysin from the enzymes mentioned, and from α-lysin as noted in the preceding paragraphs.

TABLE V

ENZYME ACTIVITY OF CRUDE, PRECIPITATED, AND PURIFIED δ-LYSIN

Enzyme	Activity		
	Crude	Precipitated*	Purified†
Coagulase	Tr.	Nil	Nil
Proteinase	Tr.	Nil	Nil
Lipase‡	35	30	Nil
Phosphatase§	0.24	0.22	Nil
Hyaluronidase‖	14	13	Nil

*At pH 4.0 with 25% (v/v) cold methanol.
†Purified by extraction with ethanol–buffer mixture as described in text.
‡Time in minutes required to cloud.
§Optical density of phenolphthalein released.
‖Reducing sugar released, reported as γ-glucose per sample.

TABLE VI

SEPARATION OF α- FROM δ-LYSIN BY FILTRATION

Fraction	Dilution	Hemolysis, D_{541}*		Units/ml
	Against human erythrocytes			
1. Original	1:100	0.211	0.217	1400
2. Original heated	1:50	0.170	0.169	450
3. Retained on filter	1:60	0.200	0.200	700
4. No. 3 heated	1:25	0.230	0.240	450
5. Filtrate	1:25	Nil	Nil	—
6. Mixed residue and filtrate Nos. 3 and 5		0.210	0.201	—
	Against rabbit erythrocytes			
7. Filtrate	1:100	0.221	0.216	—
8. Filtrate heated	1:25	Nil	Nil	—

NOTE: 5 ml crude lysin filtered through ultrafine sintered glass at 4° C. Retained material washed once with 2.5 ml water, then recovered from surface of filter by scraping with plastic "policeman" to suspend in saline–phosphate buffer, pH 6.8, and transferred by pipette to centrifuge tube. Surface of filter rewashed with buffer and added to retained material—total volume 5 ml.

*See footnote Table I.

Separation of α- and δ-Lysins by Filtration

When an attempt was made to prepare sterile lysin by filtration through ultrafine sintered-glass filters, it was found that the filtrate contained no δ-lysin. A brown-colored residue which could be recovered from the surface of the sintered plate contained the δ-lysin however. Repetition of this experiment with crude preparations clarified by centrifuging at high speed, precooled, and filtered at 4° C typically gave the results shown in Table VI. It will be seen that although not more than a trace of δ-lysin passed through the filter, recovery of this lysin from the filter surface did not exceed 50%. Since the heat-stable fraction was recovered quantitatively, loss was confined to the heat-labile portion. As filtration would not appear to introduce conditions likely to cause destruction of the heat-labile material some other explanation was sought for the observed loss of activity. The possibility was examined that a filterable fraction, inactive alone against human erythrocytes, may act synergistically with δ-lysin or a product retained by the filter. This possibility must be excluded however, since a mixture of recovered δ-lysin and filtrate did not exhibit significantly greater lytic activity than the recovered lysin employed alone. The filtrate was found to contain the α-lysin as indicated by its failure to lyse human erythrocytes and by complete loss of activity against rabbit erythrocytes upon being heated at 60° C for 10 minutes.

Discussion

The previously reported (9) partial resistance of δ-lysin to inactivation by heating at 60° C was confirmed. Moreover, it was found that contaminating α-lysin may be inactivated by heating under conditions which cause loss of only a portion of the δ-lysin present in crude preparations. The susceptibility of δ-lysin to heating at 60° C appears to vary from almost complete

resistance to 75% inactivation, depending upon the culture employed for lysin production. This would appear to explain the discrepancy between previous reports on the heat stability of this lysin ranging from resistance to autoclaving (5) to losses of 75% or more during heating at 65° C (9).

An examination of the time curve of heat inactivation revealed that a portion of the apparent δ-lysin is inactivated by heating crude lysin at 60° C for 5 minutes or less, and that continued heating for a period of 2 hours caused no further loss of activity. This would suggest existence of two different materials possessing capacity to lyse human erythrocytes: one rapidly labile and the other stable to heating at 60° C. The suggestion is supported by results of the extraction experiments. Extraction of freeze-dried, or of solvent-precipitated, crude lysin with a mixture of 75% ethanol in water or buffer resulted in recovery of heat-stable δ-lysin only. Moreover, lysin recovered in this manner was roughly equivalent, quantitatively, to the heat-stable δ-lysin present in the original crude material. Marks and Vaughan (9), likewise, found that although direct extraction of surface cultures *in situ* gave low and variable yields of δ-lysin, material obtained in this manner was stable to heating at 100° C. The above evidence points to the existence of a heat-labile and a heat-stable material possessing δ-lysin-like activity. Hence the possibility arises that two quite different lysins may be present in what has previously been designated δ-lysin. Further work is necessary, however, to clarify this point.

The observation that δ-lysin is retained on an ultrafine sintered-glass filter, while α-lysin passes through, permits the preparation of δ-lysin consisting of heat-labile and stable fractions essentially free of α-lysin. It also indicates that the physical characteristics of the two lysins (α- and δ-) differ quite markedly.

Partial purification of δ-lysin was accomplished by extracting this lysin from precipitates containing α- and δ-lysins, with 75% ethanol in water or buffer. Failure to extract the lysin with 95% ethanol does not conflict with the results of Marks and Vaughan (9), who reported solubility of this lysin in absolute ethanol. In their procedure, although absolute alcohol was employed, it was added to the bacterial cultures growing on cellophane laid over solid medium, and hence the alcohol was diluted with water present in the medium.

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AN ASSAY PROCEDURE AND PARTIAL CHARACTERIZATION OF A FACTOR IN YEAST AUTOLYSATE ESSENTIAL FOR THE GROWTH OF ACETOBACTER GLUCONICUM STRAIN 2G[1]

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Abstract

An assay procedure is described for an unknown factor in yeast autolysate essential for the growth of the assay organism, *Acetobacter gluconicum* strain 2G. The experimental evidence appears to suggest the following characteristics for the substance or substances in yeast autolysate: unaffected by acid or alkaline hydrolysis; more soluble in alcohol and acetone than chloroform; extractable by ethyl ether at pH 2.0, but not at pH 10; and growth-promoting activity is associated with material adsorbed by an anionic but not by a cationic resin. The evidence appears to indicate that the growth-promoting activity in yeast autolysate is associated with acidic substances.

During a survey of the nutritional requirements of the acetic acid bacteria, Rao and Stokes (1) found that certain fastidious strains of *Acetobacter* would not grow in a defined medium consisting of casamino acids, 10 B-vitamins, mineral salts, and glucose unless yeast autolysate was added. These authors tested a series of purified and partially purified growth factors, and concluded that these fastidious *Acetobacter* strains required new and as yet unidentified growth factors.

In the light of the above report an investigation was carried out in an attempt to characterize further the essential factor or factors in yeast autolysate. However, before this could be done, an assay procedure had to be devised which would enable the growth-promoting activity to be measured on a quantitative basis. This paper is a report of the assay procedure and a partial characterization of the yeast autolysate factor.

Methods and Experimental

Five of the fastidious strains of *Acetobacter* were obtained from Dr. C. B. van Niel, and were labelled as follows: *A. gluconicum* 1G and 2G (M.A.* 9.3 and 9.4); *A. melanogenum* 1M and 2M (M.A.* 6.3 and 6.5); and *A. rancens* 2R (M.A.* 7.1). The organisms were routinely grown on a medium which consisted of yeast extract 0.5%, potassium dihydrogen phosphate 0.3%, glucose 2.0%, and agar 2.0%, which was adjusted to pH 6.2, and autoclaved at 121° C for 15 minutes.

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*M.A. original designation in the stock culture collection of Dr. van Niel.

The nutritional studies were carried out using the medium of Rao and Stokes (1), which contains the following:

Glucose	2.0 g
Casein hydrolysate (vitamin-free)	0.5 g
Salt solution A*	0.5 ml
Salt solution B†	0.5 ml
Vitamin mixture: 40 μg each of thiamin, riboflavin, nicotinic acid, and pantothenic acid, 80 μg of pyridoxine, 10 μg of paraminobenzoic acid, 1 μg of folic acid, 0.1 μg of vitamin B_{12}, 0.04 μg of biotin, and 0.5 mg of inositol.	
Ion-free water to	100 ml
Medium adjusted to pH 6.2	

This medium was made up to double strength and dispensed in 2.5-ml amounts into 25-ml Erlenmeyer flasks and the total volume was made up to 5.0 ml. Glass or aluminum caps were used on the flasks to avoid nutritional contamination from cotton plugs (2). The flasks were autoclaved at 121° C for 15 minutes and were inoculated from a dilution blank consisting of the basal medium diluted with ion-free water, in which a small amount of growth was suspended to give a turbidity reading of 15 to 20 Klett units. One drop from a 1.0-ml pipette was used as the inoculum. This procedure was used to minimize the effect of substances which might be carried over with the inoculum from the yeast extract medium. The flasks with no additions gave low readings which attested to the fact that washing of the inoculum was not necessary. The inoculated flasks were stationary and incubated at 28° C for 5 days.

Five pounds of yeast autolysate were purchased from the Albimi Company‡ in order to have an adequate supply of the material which would last throughout the whole series of experiments.

Assay procedure.—Each of the five organisms was tested as to its response to increasing amounts of yeast autolysate. *A. gluconicum* 2G was found to give the best response over a range of 20 to 80 mg of yeast autolysate. It can be seen from Fig. 1 that there is a relationship between growth and the amount of yeast autolysate employed. In order to check the accuracy of the assay procedure, different quantities of yeast autolysate (75.0 mg, 53.0 mg, 37.0 mg, 26.0 mg, and 18.0 mg) were placed in a series of flasks, to which the basal medium was added. A standard curve was made with each experiment from which the quantity of yeast autolysate was determined, and the percentage error was calculated. From the results summarized in Table I, it becomes evident that the mean percentage error for this assay procedure appears to be quite similar from 75.0 mg to 26.0 mg, but the error sharply increases at the 18.00 mg level.

*Salt solution A

KH_2PO_4	50 g
K_2HPO_4	50 g
H_2O	500 ml

†Salt solution B

$MgSO_4.7H_2O$	20 g
NaCl	1 g
$FeSO_4.7H_2O$	1 g
HCl, concentrated	1 ml
H_2O	500 ml

‡Brooklyn, New York.

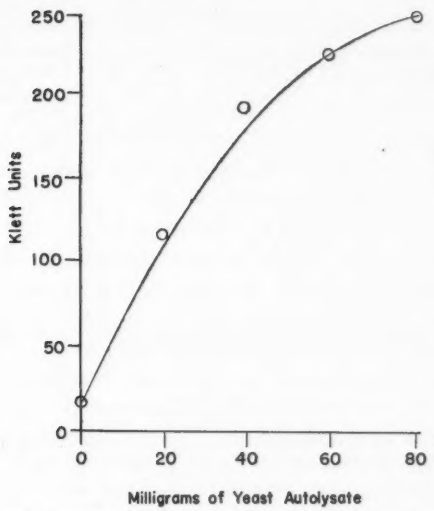

FIG. 1. The growth response of *Acetobacter gluconicum* 2G to increasing amounts of yeast autolysate.

TABLE I

THE ERROR OF THE MICROBIOLOGICAL ASSAY PROCEDURE FOR THE YEAST AUTOLYSATE FACTOR

Weight of yeast autolysate, mg	Mean % error	Number of experiments
75.0	6.6	12
53.0	7.2	14
37.0	7.8	14
26.0	8.3	14
18.0	11.00	7

It becomes evident from these results that the error in these determinations is acceptable for microbiological assay.

Characteristics of the Yeast Autolysate Factor

The stability of the activity in yeast autolysate to 0.1 N H_2SO_4 *and 0.1 N* KOH *at 121° C for 15 minutes.*—Five per cent solutions of yeast autolysate were made up in the following diluents: distilled water, 0.1 *N* H_2SO_4, and 0.1 *N* KOH. These solutions were autoclaved at 121° C for 15 minutes. The pH values of the solutions containing the alkali and the acid were readjusted to 6.9, the original pH before treatment, and assayed for growth-promoting activity. It becomes evident from the results in Table II that the growth-promoting ability of the yeast autolysate when treated with an acid or with an alkali are very similar. When these values are compared with the heated distilled water control, the treated values are slightly lower, but the significance of these differences appears doubtful. Therefore, it appears that the yeast autolysate factor is resistant to acid and alkaline hydrolysis.

TABLE II

THE STABILITY OF THE GROWTH-PROMOTING FACTOR IN YEAST AUTOLYSATE 0.1 *N* H_2SO_4 AND 0.1 *N* KOH AT 121° C FOR 15 MINUTES

Weight of material tested, mg	Specific activity*		
	0.1 *N* H_2SO_4	0.1 *N* KOH	Distilled water
40	1.00	0.90	1.16
50	0.84	0.84	1.06
60	1.00	0.87	1.20
Average	0.94	0.87	1.12

*Specific activity: The increase or decrease of growth-promoting ability associated with a given weight of material when compared with untreated yeast autolysate with a value of 1.0.

TABLE III

THE GROWTH-PROMOTING ABILITY ASSOCIATED WITH MATERIALS EXTRACTED BY VARIOUS SOLVENTS

	Specific activity*
Trial 1	
Ethanol extract	1.25
Ethanol residue	0.33
Trial 2	
Ethanol extract	1.33
Ethanol residue	0.62
Acetone extract	1.05
Acetone residue	0.48
Chloroform extract	0.42
Chloroform residue	0.98

*Specific activity: The increase or decrease of growth-promoting ability associated with a given weight of material when compared with untreated yeast autolysate with a value of 1.0.

TABLE IV

THE GROWTH-PROMOTING ABILITY ASSOCIATED WITH MATERIALS EXTRACTED AT pH 2.0 BY ETHYL ETHER

	Specific activity*
Trial 1	
Extract	3.70
Residue	0.45
Trial 2	
Extract	4.60
Residue	0.75

*Specific activity: The increase or decrease of growth-promoting ability associated with a given weight of material when compared with untreated yeast autolysate with a value of 1.0.

The growth-promoting ability of substances extracted from yeast autolysate by various solvents.—Extractions of the dry yeast autolysate were made using 95% ethyl alcohol, acetone, and chloroform, using a Soxhlet extractor. The results of these procedures are summarized in Table III. It appears that the yeast autolysate factor is more soluble in ethanol and acetone than in chloroform. Ethanol appears to extract slightly more activity than acetone, but in either case, the concentration of the growth-promoting activity was only slightly higher than the untreated yeast autolysate.

Continuous ether extraction of yeast autolysate solutions at pH 2.0 and 10.0.—Five per cent yeast autolysate solutions were subjected to continuous ether extractions at pH 2.0 and 10.0. The pH of the extract and the residues were adjusted to the original pH before treatment, 6.9, and were assayed. The results of these procedures are found in Table IV. It becomes evident that growth-promoting activity was associated with the materials extracted at pH 2.0. There was no activity after extraction at pH 10.0. These data indicate that the activity is associated with the acid fraction of yeast autolysate.

The growth-promoting ability of substances adsorbed from yeast autolysate by the use of ion-exchange resins.—Two types of ion-exchange resins were used, IRA-45 and IRC-50. Solutions of yeast autolysate were passed through resin columns 7×40 cm. The pH of the solutions were readjusted where necessary, and assayed. The results of these experiments are summarized in Table V, and it was found that materials with growth-promoting activity were adsorbed by the anionic but not by the cationic resin.

These results agree with those obtained by ether extraction associating activity with the acid fractions.

TABLE V

THE PREFERENTIAL ADSORPTION OF MATERIALS FROM YEAST AUTOLYSATE POSSESSING GROWTH-PROMOTING ABILITY BY AN ANIONIC RESIN, IRA-45, BUT NOT BY A CATIONIC RESIN, IRC-50

Ion-exchange resin	Volume assayed, ml	Specific activity* of effluent after adsorption
IRC-50 (cationic)	0.5	2.2
	1.0	1.9
	1.5	1.6
	2.0	1.6
IRA-45 (anionic)	0.5	0
	1.0	0
	1.5	0
	2.0	0

*Specific activity: The increase or decrease of growth-promoting ability associated with a given weight of material when compared with untreated yeast autolysate with a value of 1.0.

Discussion

The results obtained by the alkaline and acid hydrolysis of yeast autolysate at 121° C for 15 minutes indicated the growth-promoting ability of yeast autolysate for *A. gluconicum* 2G was quite stable. Alkaline hydrolysis causes degradative changes with carbohydrates and racemization of amino acids. The fact that amino acids were readily available in the growth medium from the hydrolyzed casein implied that some other class of compound was required by the organism.

Since ethanol appeared to extract more growth-promoting activity for the assay organism, *A. gluconicum* 2G, than chloroform, this evidence is suggestive that the extracted material from the dry yeast autolysate might be polar in nature. Furthermore, the substances extracted from a yeast autolysate solution at pH 2.0 with ethyl ether possessed growth-promoting activity while none was obtained at pH 10.0. This observation, plus the fact that growth-promoting activity was associated with materials adsorbed by an anionic but not with a cationic resin, indicated that growth-promoting activity was associated with acidic substances.

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UTILIZATION OF ACETATE, PYRUVATE, AND CO_2 BY PENICILLIUM DIGITATUM[1]

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Abstract

The utilization of C^{14} specifically labelled pyruvate, acetate, and CO_2 by proliferating *Penicillium digitatum* cells has been studied by means of a series of time-course experiments. Pyruvate appeared to be utilized by way of acetate and the latter was in turn utilized mainly via the TCA processes. Degradation studies on aspartic acid and glutamic acid isolated from cells grown on acetate-1-C^{14} or $C^{14}O_2$ indicated that CO_2 can be fixed in this organism by means of a two-stage process onto a "C_2 unit"; the latter is thought to be derived from glyoxalic acid produced in the "glyoxalate bypass" pathway.

Introduction

Recent interest in the biosynthetic mechanism of ethylene in *Penicillium digitatum* (1, 10) has led the authors to study the basic metabolic processes of carbohydrates in this organism. Previous nutritional studies (6) revealed that *P. digitatum* can utilize most of the simple carbohydrates such as hexoses and pentoses, citric and malic acids to some extent, but not fumaric, tartaric, lactic, oxalacetic, and acetic acids as growth supporting carbon sources.

In order to understand the over-all metabolism of carbohydrates in an organism, it is often desirable to identify first the nature of the oxidative mechanism for the breakdown intermediates of carbohydrates as well as some of the basic biosynthetic pathways functioning in the proliferating cells. In the present work, C^{14} specifically labelled pyruvate, acetate, and $C^{14}O_2$ were used as test substrates in a series of time-course experiments on the cellular combustion of these compounds. The incorporation of labelled acetate and CO_2 into amino acids was also investigated and the isotopic distribution patterns of aspartic acid and glutamic acid derived from these two substrates were obtained by degradation studies. The findings in these studies revealed the important roles played by the tricarboxylic acid cycle and a CO_2 fixation process involving an "active C_2 unit" for the respiratory and biosynthetic functions in this organism.

Materials and Methods

The organism *P. digitatum* (Sacc.) was isolated from lemon fruits. Stock cultures were made on potato-glucose-agar slants at room temperature for a week. Spores for inocula were then obtained from the test-tube slants

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and used to inoculate 500 ml of a nutrient medium (20) contained in a three-neck flask. Incubation was allowed to continue for 3 to 4 days under vigorous aeration. After that, cells were harvested by centrifugation, washed well, and resuspended in a "carbon-source-free medium," which is essentially the same as that mentioned above but with all the carbonaceous compounds excluded.

Time-Course Studies of Substrate Utilization

The apparatus used in these studies is essentially the one described previously (18), which consists of a battery of assemblies, each consisting of an air-purification system, flowmeter, three-necked incubation flask, and CO_2 trap, connected together by means of a bypass manifold. In the present work, three series of experiments have been carried out: (1) 0.15 millimoles of either acetate-1-C^{14} or acetate-2-C^{14} (125 μc) utilized by 200 ml of the cell suspension containing 200 mg of cells (dry weight); (2) trace amounts of either acetate-1-C^{14} or acetate-2-C^{14} (1.5 μc) and 90 mg of glucose utilized by 250 ml of the cell suspension containing 250 mg of cells; (3) trace amounts of pyruvate-1-, 2-, or 3-C^{14} (0.5 μc) and 90 mg of glucose utilized by 250 ml of the cell suspension containing 250 mg of cells. Samples of respiratory CO_2 were collected every hour for radioactive assay, and at the end of each experiment, cells and incubation medium were separated by centrifugation and processed for the measurement of their respective radioactivity. Portions of the cells were also hydrolyzed and the resulting hydrolyzate subjected to paper chromatographic separation of the amino acids.

Incorporation of Acetate into Cellular Constituents

In the incorporation studies, experiments similar to those described in the foregoing section but larger in scale were carried out. Usually 500 ml of the cell suspension containing 500 mg of cells were used in each flask and the quantity of acetate, the sole carbon source, was also increased proportionally. Experiments were terminated at 4 hours by the addition of HCl to give the medium a pH of 1.0. Cells were harvested by centrifugation, washed thoroughly with water and alcohol, and dried over P_2O_5. The radioactivity in the respiratory CO_2, cells, and culture medium was determined in the usual manner.

The dried cells were hydrolyzed by autoclaving with 20% HCl in a Carius tube for 9 hours. The resulting hydrolyzate was filtered through a sintered glass filter to remove humin and evaporated repeatedly *in vacuo* to dryness to remove free HCl. Finally, the residue was taken up in water and extracted exhaustively with ether in a liquid–liquid extractor to remove any fatty material. The radioactivity of each fraction was determined either by direct plating technique or wet combustion to CO_2 and counted as $BaCO_3$.

Fixation of $C^{14}O_2$

The apparatus and procedures used in the study of CO_2 fixation were essentially the same as described in a previous paper (2). Two and one-half grams of washed mold suspended in 1000 ml nutrient medium containing 10 millimoles of glucose as the carbon source were exposed to 0.43 millimoles of $C^{14}O_2$

having a specific activity of 5 mc per millimole in a closed system. Incubation was continued for 7 hours with continuous shaking. Samples of the cell suspension were taken periodically and the radioactivity of cells and medium were assayed in the usual manner. At the end of the experiment, the cells were processed and hydrolyzed in the manner described previously. C^{14} contents of individual amino acids in the hydrolyzate was determined by means of paper chromatography and scanning counting with a gas-flow paper strip counter.

Isolation of Aspartic and Glutamic Acids

Aspartic and glutamic acids were separated from the respective cell hydrolyzates by means of ion-exchange column chromatography (8), and isolated as free amino acid after the addition of an appropriate amount of the respective carrier compound. Purity of each acid was established by paper chromatography.

Degradation of Aspartic and Glutamic Acids

Aspartic acid was degraded by: (i) total combustion for the specific activity of whole molecule; (ii) ninhydrin decarboxylation for the specific activity of carbon atoms 1 and 4; (iii) nitrous acid deamination (5) followed by sulphuric acid decarbonation to yield C-1 as carbon monoxide, which was in turn oxidized to CO_2 for radioactivity assay (16); (iv) nitrous acid deamination followed by permanganate oxidation to give acetaldehyde equivalent to carbon atoms 2 and 3 (3). A portion of the latter was isolated as its 2,4-dinitrophenylhydrazone for radioactive assay and the remainder was converted to iodoform for the determination of the specific activity of C-3. Specific activities of carbon atoms 2 and 4 were obtained by difference.

The isotopic distribution pattern of glutamic acid was obtained by: (i) total combustion for the specific activity of the whole molecule; (ii) ninhydrin decarboxylation for the specific activity of alpha carboxyl carbon atom; (iii) Schmidt's decarboxylation to give the specific activity of the gamma carboxyl carbon atom; (iv) the activity of the middle carbon atoms was estimated by difference.

Results and Discussion

The interval radiochemical recovery of substrate activity in respiratory CO_2 from proliferating *P. digitatum* cells metabolizing C^{14} specifically labelled acetate or pyruvate in the presence of ordinary glucose is shown in Figs. 1 and 2 respectively. In a separate experiment, labelled acetate was used as the sole carbon source from which resulted a pattern closely resembling that given in Fig. 1. From these data, it is apparent that insofar as the combustion of these substrates to CO_2 is concerned, pyruvate is first decarboxylated oxidatively giving rise to acetate which is in turn metabolized by way of a sequence of processes involving the preferential combustion of C-2 of pyruvate or C-1 of acetate. The latter process is probably in the nature of the classical tricarboxylic acid cycle (TCA) in view of the similarity observed between the utilization patterns of acetate in this organism and that in *Penicillium chrysogenum* (11) and the reported TCA activity in the latter organism (7).

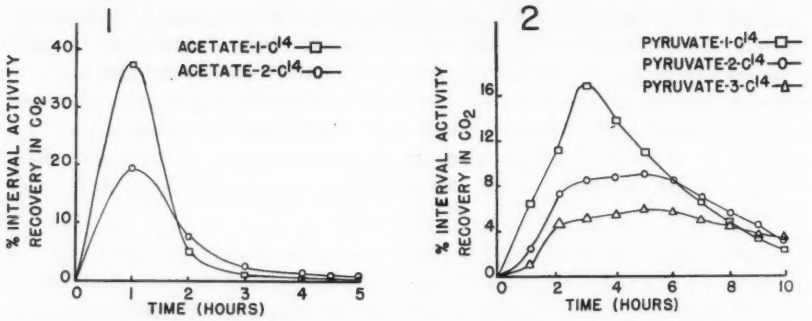

FIG. 1. Interval radiochemical recovery in respiratory CO_2 from 44-hour-old culture of *P. digitatum* grown on glucose in the presence of tracer amount of specifically C^{14}-labelled acetate.

Substrate: acetate-1-C^{14}, acetate-2-C^{14}.

Time in hours: time for medium glucose disappearance, 3–4 hours. Weight of cells per flask, 250 mg. Air-flow rate per flask, 400 ml per minute. Level of glucose per flask, 90 mg. Level of radioactivity per flask, 1.5 μc. Temperature, 30° C.

FIG. 2. Interval radiochemical recovery in respiratory CO_2 from 44-hour-old culture of *P. digitatum* grown on glucose in the presence of tracer amount of specifically C^{14}-labelled pyruvate.

Substrate: pyruvate-1-C^{14}, pyruvate-2-C^{14}, pyruvate-3-C^{14}.

Time in hours: time for medium glucose disappearance, 3–4 hours. Weight of cells per flask, 250 mg. Air-flow rate per flask, 400 ml per minute. Level of glucose per flask, 90 mg. Level of radioactivity per flask, 0.5 μc. Temperature, 30° C.

Table I gives the radiochemical recovery of substrate activity in respiratory CO_2, cellular constituents, and fermentation products from proliferating cells metabolizing the labelled acetate or pyruvate. It is noted that considerably more activity has been incorporated into cellular constituents from C-2 of acetate or C-3 of pyruvate. This fact is in line with the operation of TCA cyclic processes and related reactions in producing various carbon skeletons for the biosynthesis of cellular compounds, particularly that of amino acids. This is further supported by the time-course study of the incorporation of acetate-1-C^{14} into cellular amino acids. As shown in Table II, aspartic acid and glutamic acid, being related more directly to acetate via TCA cycle, were

TABLE I

DISTRIBUTION OF SUBSTRATE ACTIVITY IN *P. digitatum* UTILIZING C^{14}-LABELLED PYRUVATE OR ACETATE

Substrate	Radiochemical recovery, %		
	Resp. CO_2	Cells	Fermentation products (medium)
Pyruvate-1-C^{14}	87	7	8
Pyruvate-2-C^{14}	69	29	6
Pyruvate-3-C^{14}	46	40	10
Acetate-1-C^{14}	48	20	2
Acetate-2-C^{14}	35	37	2

labelled very rapidly. Labelling subsequently occurred in the rest of the amino acids at about four hours after the administration of the labelled acetate into the incubation medium.

The important role played by CO_2 in the over-all metabolism of this organism is demonstrated by the time-course study of the incorporation of $C^{14}O_2$ into cellular amino acids shown in Table II. It is noted that 30 minutes after the exposure of cells to $C^{14}O_2$, aspartic and glutamic acids became labelled, evidently as the result of the operation of a CO_2 fixation mechanism. Later in the experiments, other amino acids related to the foregoing two compounds, such as proline, glycine, threonine, tyrosine (12), lysine, and isoleucine were also labelled. Inasmuch as inactive glucose was present throughout the experiment, it is not surprising to find that alanine and other amino acids (valine and leucine) known to be derived from pyruvate, remained unlabelled even at the end of the experiment. It is also noteworthy that $C^{14}O_2$ was incorporated into tyrosine but not phenylalanine. This fact might reflect that some fundamental differences exist in the respective biosynthetic schemes of these amino acids. Similar findings have been reported in the case of bakers' yeast (13).

TABLE II

INCORPORATION OF C^{14} INTO CELLULAR AMINO ACIDS OF *P. digitatum* UTILIZING $C^{14}O_2$ OR ACETATE-1-C^{14}

	$C^{14}O_2$ plus unlabelled glucose		Acetate-1-C^{14}		
	Time, hours		Time, hours		
Amino acids	½	5¾	¼	2	4
Aspartic acid	++	++++	+	++	++++
Glutamic acid	++	++++	+	++	++++
Proline		+			++
Glycine and serine		+			++
Alanine					++
Threonine		+			+
Phenylalanine					+
Tyrosine		+			+
Valine					++
Leucine					+
Isoleucine		+			+
Arginine		++			++
Lysine		+			+

In order to elucidate further the mechanism of the utilization of acetate and CO_2 in the biosynthesis of amino acids in this organism, two key amino acids, i.e. aspartic acid and glutamic acid, were isolated from cells metabolizing either acetate-1-C^{14} alone or $C^{14}O_2$ in conjunction with inactive glucose. The isotopic distribution patterns of the amino acids in these experiments are given in Table III.

In the case of aspartic acid, both carboxyl carbon atoms were found to be heavily labelled from either acetate-1-C^{14} or $C^{14}O_2$.

TABLE III

DISTRIBUTION OF C^{14} IN ASPARTIC ACID AND GLUTAMIC ACID FROM *P. digitatum* UTILIZING $C^{14}O_2$ OR ACETATE-1-C^{14}

Carbon atoms	Labelled substrate	
	$C^{14}O_2$, % of total	Acetate-1-C^{14}, % of total
Aspartic acid		
1. COOH	24	25
2. $CHNH_2$	18	36
3. CH_2	0	0
4. COOH	58	40
Glutamic acid		
1. COOH	85	28
2. $CHNH_2$	13	38
3. CH_2		
4. CH_2		
5. COOH	2	33

This is not surprising since the operation of either the conventional tricarboxylic acid cyclic processes or the malate synthetase reaction (19) will result in the exclusive labelling of carboxyl carbon atoms of oxalacetate, hence aspartate, from acetate-1-C^{14}. Fixation of $C^{14}O_2$ of the $C_3 + C_1$ type in conjunction with the possible equilibration of the C_4 acid so formed with a symmetrical C_4 acid could have accounted for the appearance of $C^{14}O_2$ activity in the carboxyl carbon atoms of aspartic acid. However, the detection of significant amount of C^{14} from either labelled substrate in the amino carbon atoms, together with the finding that the β-carboxyl is labelled considerably heavier than the α-carboxyl of this amino acid, point to the involvement of some other pathways in the biosynthesis of aspartic acid in this organism.

Earlier, the incorporation of C-1 of acetate into C-2 of aspartic acid has been observed by Cutinelli *et al.* in *Rhodospirillium rubrum* (4) and by Tomlinson in *Clostridium kluyveri* (14). In either case the findings were interpreted as indicative of the operation of a two-stage CO_2 fixation process which involves (1) the condensation of CO_2 onto a two-carbon unit related to acetate and (2) the subsequent CO_2 fixation by the C_3 compound so formed. The latter reaction gives rise to a C_4 acid presumably oxalacetate, although the exact nature of the C_2 unit was not elaborated. When $C^{14}O_2$ was used as substrate to these two organisms, contrary to the findings in the present experiment, labelling was detected only in the carboxyl carbon atoms of aspartic acid, although a heavier label in favor of β-carboxyl carbon atoms was noted in the case of *R. rubrum*. It is also noteworthy though that in the *R. rubrum* experiment, considerable C^{14} from $C^{14}O_2$ was incorporated into C-2 of threonine, which is known to be closely related to oxalacetate.

In view of the resemblance among those findings in *R. rubrum* and *C. kluyveri* as compared to the present work, it is reasonable to believe that the proposed CO_2 fixation of the $C_2 + C_1$ type may also be operative in *P. digitatum*.

However, in the present work the labelling of C-2 of aspartic acid from either acetate-1-C^{14} or $C^{14}O_2$ also provides some information as to the nature of the "C_2 compound" in the CO_2 fixation process in question. In the experiment with *R. rubrum* (4) Cutinelli *et al.* speculated that the "C_2 compound" is closely related to acetate and is probably a reduction product of the latter. Results obtained in the present experiment revealed that CO_2 can be incorporated readily into C-1 of the "C_2 compound" but not into C-1 of acetate as indicated by the very low C^{14} content in the γ-carboxyl carbon atoms of glutamic acid (Table III) in the $C^{14}O_2$ experiment. In light of the recent report on the functions of the "glyoxalate bypass" pathway in microbial biosynthesis (9) it appears that the "C_2 compound" involved in the CO_2 fixation process may have been derived from glyoxalic acid generated in the isocitratase reaction. Since the carboxyl carbon atom of glyoxalic acid is equivalent to C-4 of oxalacetate prior to the citrate condensation, an avenue is thus provided for the incorporation of C^{14} from either acetate-1-C^{14} or $C^{14}O_2$ into the amino carbon atom of aspartic acid as observed in the present work. Although the exact nature of the "C_2 compound" cannot be defined in the present work, one might speculate on the basis of the known mechanisms of other CO_2 fixation processes that the "C_2 compound" could have been a reduction product of glyoxalic acid such as glycolaldehyde. Evidently, extensive equilibration of the aspartic acid precursor with a symmetrical C_4 acid is not realized in the present experiment, since from either labelled substrate no C^{14} was detected in C-3 of aspartic acid and considerably heavier labelling was observed in the β-carboxyl of aspartic acid. The findings thus necessitate the unsymmetrical nature of the "C_2 compound" engaged in the CO_2 fixation process, which implies that if glycolaldehyde is involved in the biosynthetic scheme, it is probably occurring in a form invulnerable to randomization such as the phosphorylated moiety.

The isotope distribution patterns of glutamic acid samples isolated from cells grown on either acetate-1-C^{14} or $C^{14}O_2$ and glucose provided additional evidence for the foregoing conclusions. Thus, $C^{14}O_2$ was found to be incorporated heavily into the α-carboxyl carbon atom of glutamic acid. This fact is in line with the operation of the oxalacetate–citrate–oxalosuccinate sequence in the TCA cyclic process for the formation of α-ketoglutaric acid, presumably the precursor of glutamic acid. The small amount of $C^{14}O_2$ activity in the middle carbon atoms was undoubtedly derived from the C_4 moiety in the citrate condensation step of the TCA cycle. The minute amount of C^{14} in the γ-carboxyl carbon atom is in sharp contrast to the reported heavy labelling from $C^{14}O_2$ in *C. kluyveri* (15) but resembles closely the findings in *R. rubrum* (4). With acetate-1-C^{14} as substrate, although heavier labelling is observed in the γ-carboxyl carbon atom as compared with that in the α-carboxyl carbon atom, the ratio of γ-COOH/α-COOH is close to unity instead of a ratio of 2 as observed in the case of yeast (17), which is known to have extensive operation of TCA cyclic processes.

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STAPHYLOCOCCAL TOXINS

IV. FACTORS AFFECTING HEMOLYSIS BY β-LYSIN[1]

A. W. JACKSON AND DAVID MAYMAN

Abstract

The effects of a number of factors upon hemolysis by staphylococcal β-lysin have been examined. It has been found that crude β-lysin loses much of its hemolytic activity upon dialysis, but activity is restored by addition of magnesium or manganese ions. At optimum levels (0.01–0.015 *M*) of magnesium, hemolytic activity may be 35 times as great as that of dialyzed lysin. Lytic activity is maximal at neutral reactions but decreases sharply below pH 6.8. Addition of gelatin or albumin to the system causes β-lysin to exhibit increased hemolytic activity. This increase is not due, however, to protection of the lysin against spontaneous loss of activity in dilute solution. A typical time–hemolysis curve is sigmoid and a family of similar curves distributed along the time axis may be obtained with a series of different lysin concentrations. Relative β-lysin concentrations may be determined under defined conditions of erythrocyte, buffer, gelatin, and magnesium ion concentrations and at pH 7.0 employing concentrations of lysin which cause not less than 10, and not more than 75–80% hemolysis.

Introduction

It has been recognized since 1922 (12) that certain strains of *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) produce an unusual lysin which causes lysis of goat erythrocytes only upon being cooled, after a period of warm incubation with the lysin. This is the "hot–cold" lysin of Bigger, Boland, and O'Meara (1) and the β-lysin of Glenny and Stevens (5). Erythrocytes of the ox and sheep have been reported (11) to be highly susceptible to lysis by this lysin, whereas erythrocytes of human origin (9, 11) were found to be slightly susceptible, and those from a number of other animal species (11) quite resistant.

Although the "hot–cold" lysin of *S. aureus* has been recognized for some time, as noted above, a detailed examination of the action of this lysin on erythrocytes has not been published; consequently, such a study was undertaken to define characteristics of the lysin which may assist in its identification, and to determine conditions under which a simple, direct test for hemolytic activity may be conducted.

S. aureus culture, J32*a*, which produces monovalent β-lysin, was employed in the present study as a source of lysin. We are indebted to Professor C. E. Dolman, Department of Bacteriology and Immunology, University of British Columbia, who kindly provided this culture.

Materials and Methods

Cultures

S. aureus culture No. J32*a* was examined for lysin production by growing individual colonies on sheep blood agar plates. No detectable direct lysis

[1]Manuscript received May 9, 1958.
Contribution from the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

was produced during incubation at 37° C, but clear zones of hemolysis surrounding colonies appeared after cooling and hence this culture was considered to be an exclusively β-lysin producer (3).

Production of Lysin

Cultures were grown on cellophane sheets laid over solid agar as described for δ-lysin production (7). Seeded plates were incubated in a mixture of 25% carbon dioxide in oxygen at 37° C for 24 hours (2). The lysin was recovered by washing cells and lysin from the plates, and removal of cells by centrifuging in the manner described for δ-lysin (7).

Determination of "Hot–Cold" Hemolytic Activity

One milliliter of appropriately diluted lysin is added to 5 ml of saline–phosphate buffer (6), pH 7.0, in a test tube and warmed to 37° C in a water bath. One milliliter of a 7% suspension of thrice washed sheep erythrocytes is then added to the tube and mixed with the lysin to give a 1% suspension of erythrocytes. After the indicated period of incubation at 37° C the tubes are placed in a cold water bath at 4° C and cooled for 15 minutes, unless otherwise stated. Contents of the tubes are then transferred to centrifuge tubes (15-ml capacity) and the unhemolyzed erythrocytes removed by centrifugation. A "hot" lysis, where required, is conducted as above with the exception that the contents of the tubes are poured into previously warmed centrifuge tubes and centrifuged immediately after the required period of incubation at 37° C. The released hemoglobin is diluted—1 ml with 6 ml of a solution prepared by mixing 3 volumes of 0.1% sodium carbonate and 1 volume of 0.1% sodium bicarbonate—and the optical density of the diluted solution is determined at 541 mμ employing matched 18-mm absorption tubes in a Coleman Jr. spectrophotometer. The extent of hemolysis is determined by comparing the value obtained above with that for complete hemolysis.

The complete hemolysis value is obtained by adding 1 ml of a 7% suspension of erythrocytes to 6 ml of distilled water and diluting 1 ml of the resulting suspension in 6 ml of the sodium carbonate solution, the optical density of which is determined as above. A 1% suspension of cells prepared as described above was found to contain 2.4×10^8 erythrocytes per ml and when diluted and lysed as described gave an optical density of 0.33 at 541 mμ.

Erythrocytes from different individual animals varied considerably in susceptibility to β-lysin, the most sensitive giving lysin activities 1.5 times higher than the least sensitive. It was also noted that blood stored for periods in excess of 3 weeks showed a marked decrease in susceptibility to lysis by this lysin. As a consequence, comparative tests were conducted at one time employing aliquots from a single suspension of washed erythrocytes.

Results

Effect of Duration of Cooling on "Hot–Cold" Lysis

The typical experiment (10) conducted to determine the hemolytic activity of β-lysin consists of incubating erythrocytes and lysin together at 37° C for

1 hour followed by storage of the suspension at 4° C or at room temperature overnight to allow "cold" hemolysis to occur. The tubes are then examined for extent of hemolysis. A rapid method of determining β-lysin activity requires that all steps in the procedure be reduced to the shortest possible time, hence the effect of duration of cooling upon hemolysis was examined. The results shown in Fig. 1 are those of a typical experiment in which erythrocyte suspensions exposed to three different concentrations of lysin for a period of 25 minutes were cooled by immersion in a water bath held at 4° C. It appears that erythrocytes exposed to this lysin during a suitable period of incubation at 37° C lyse quite rapidly upon being cooled, the rate of lysis being most rapid with the first 5 minutes of cooling. After the initial rapid lysis a slower but definite rate of hemolysis occurred; after 15 minutes cooling this rate appeared to be quite similar for the different concentrations of lysin employed. As the rate of lysis at this point was low and, consequently, slight errors in the period of cooling would not materially influence the degree of lysis attained, a cooling period of 15 minutes was arbitrarily selected for all further work.

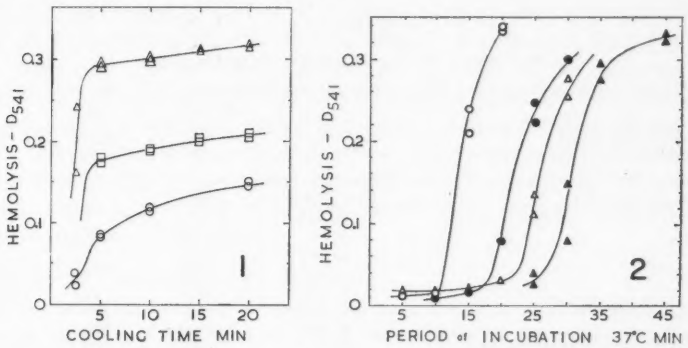

FIG. 1. Effect of cooling on hemolysis by β-lysin at three different concentrations of lysin.

FIG. 2. Effect of time of incubation at 37° C and of lysin concentration on hemolysis at four different concentrations of lysin.

Effect of Time of Incubation and of Lysin Concentration on Hemolysis

The effect of varying β-lysin concentration upon hemolysis was studied employing different periods of incubation at 37° C followed by cooling at 4° C for 15 minutes. The results obtained are shown in Fig. 2. It will be noted that varying the concentration of lysin employed shifts the curves along the time axis but does not materially alter their shape.

The typical curve shows a lag period varying in duration depending upon the concentration of lysin employed, and in which very little hemolysis occurs. Following the lag period, hemolysis becomes quite rapid and is more than 75% complete within a period of 5 minutes additional incubation at 37° C (total hemolysis, $D_{541} = 0.330 \pm 5$). Thus slight variations in the period allowed for the warm incubation will introduce perceptible change in extent of resulting

hemolysis. It should be noted that the observed lysis is that which occurs after cooling the incubated lysin–erythrocyte mixture at 4° C for 15 minutes.

Effect of Metallic Ions on Hemolysis by β-Lysin

It was observed during some preliminary tests with β-lysin that dialyzed crude preparations or partially purified preparations showed almost complete lack of hemolytic activity. This suggested the need by the lysin for some activating ion or possibly a reducing substance for maximum hemolytic activity to occur. Trials showed that magnesium sulphate caused a marked increase in the hemolytic activity of dialyzed lysin preparations. The effects of magnesium, manganese, and calcium sulphates are shown in Table I. The data indicates that the addition of 0.001 *M* magnesium sulphate causes a marked increase in the hemolytic activity of the lysin, and that maximum lytic activity is obtained with addition of this salt at 0.01 *M* concentration. With optimum levels of magnesium ion, lytic activity is approximately 35 times that observed with the dialyzed lysin alone. Magnesium chloride was found to be as effective as the sulphate and magnesium sulphate added without lysin was non-lytic,

TABLE I

ACTIVATION OF β-LYSIN WITH METALLIC IONS

Salt employed	Final concn., *M*	Dilution of β-lysin	% lysis†	Units/ml
Crude	(Non-dialyzed)	1:50	45	340
Control	(Dialyzed)	1:25	11	120
$MgSO_4$	0.001	1:500	9	2270
	0.005	1:500	51	3500
	0.009	1:500	62	3745
	0.01	1:500	76	4130
	0.015	1:500	76	4130
$MgCl_2$	0.015	1:500	76	4130
$MgSO_4$	0.015	No lysin	Nil	—
Citrate 0.01 *M* + 0.01 *M* $MgSO_4$		1:400	Nil	—
$MnSO_4$*	0.015	1:900		6300
$MgSO_4$*	0.015	1:450	20	2300
$CaSO_4$*	0.015	1:200	Nil	—
$MgSO_4$	0.015	1:600	32	3530
$MgSO_4$	0.01	1:2000	60	14,840
Versene	0.14%	Not diluted	Nil	—

*In Tris + saline buffer (0.05 *M* tris(hydroxymethyl)aminomethane plus 0.13 *M* sodium chloride; pH adjusted to 6.8).
†Average of duplicates.

indicating that magnesium ion is responsible for the effects observed with the sulphate and that these effects are an example of activation of the lysin by magnesium ion. As would be expected, addition of citrate, which forms a slightly dissociated salt with magnesium, markedly reduces the hemolytic activity of β-lysin. Furthermore, a dialyzed preparation of lysin, which exhibits an activity of approximately 15,000 hemolytic units per ml in the presence of optimum concentrations of magnesium ion, shows no activity when employed without dilution in the presence of versene.

Owing to the insoluble nature of the phosphates of calcium and manganese, these ions were added in buffers prepared from sodium chloride plus tris-(hydroxymethyl)aminomethane (Tris) as a buffering agent. Manganese was found to possess effects similar to magnesium in this system, whereas calcium was ineffective. Magnesium sulphate incorporated in Tris buffer was found to be somewhat less effective than the same ion incorporated in the usual phosphate–saline buffer. Thus the effects of manganese cannot be due to Tris buffer, nor is the complete lack of β-lytic activity in the presence of calcium ions likely ascribable to the use of Tris buffer. Magnesium sulphate at 0.01 *M* concentration was incorporated in the saline–phosphate buffers for all later tests. The salt was added without change in concentration of the remaining constituents and buffers were readjusted to pH 7.0 after addition of magnesium sulphate.

Effect of pH on Hemolysis by β-Lysin

The effect of pH upon the rate of hemolysis by β-lysin was examined since this variable is an important one in biological systems. Saline–phosphate buffers of different pH levels but with calculated equi-ionic strength (6) were prepared; these contained 0.02 *M* magnesium sulphate to replace the usual saline–phosphate buffer. Neutralized physiological saline solution was employed as a diluent for lysin and as a suspending agent for the erythrocytes. The optimum pH for hemolysis by β-lysin occurs in the region of neutrality (Fig. 3). At pH levels below neutrality there is a marked reduction in hemolytic activity, whereas, at levels above the optimum, lytic activity is less sharply reduced. As a consequence of this observation all further tests were conducted at pH 7.0.

Effect of Lysin Concentration on Hemolysis

In order to utilize degree of hemolysis as a measure of lysin activity, it is necessary to examine the relationship between lysin concentration and extent of resulting hemolysis at constant time of incubation. Increasing aliquots of a suitably diluted sample of β-lysin were added to tubes and made up to 6 ml

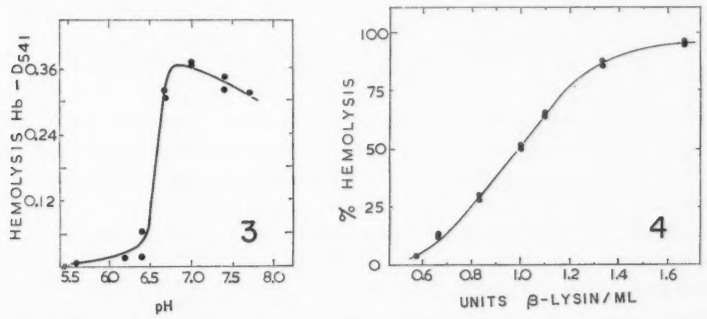

FIG. 3. Effect of pH on hemolysis by β-lysin.
FIG. 4. Relationship of lysin concentration to hemolysis. Incubation at 37° C for 25 minutes.

with buffers. This was done in duplicate and all materials were diluted in saline–phosphate buffer, pH 7.0, containing 0.01 *M* magnesium sulphate. The remainder of the hemolysis test was conducted as usual. It will be seen (Fig. 4) that the relationship between extent of hemolysis and lysin concentration is linear between approximately 10 and 75% hemolysis. With the higher concentration of lysin employed hemolysis was over 95% complete.

In keeping with the procedures generally employed, a hemolytic unit is defined arbitrarily as the quantity of β-lysin which causes 50% hemolysis of 1 ml of a 1% suspension of sheep erythrocytes under the conditions employed in the experiments described above. The quantities of lysin employed in this experiment were converted to units by assigning a value of 1 unit to the quantity of lysin which causes 50% hemolysis. Activity of the lysin sample in terms of hemolytic units per milliliter is calculated from the following equation:

$$\text{units activity/ml} = D \times (1/V_1) \times V_e \times U,$$

where D = dilution of lysin,
V_1 = milliliters of diluted lysin employed,
V_e = total volume in milliliters of mixed components (volume of 1% erythrocytes),
U = hemolytic units determined from Fig. 4.

Effect of Glucose and of Proteins on Hemolysis

Among the substances which may affect rate of hemolysis by a lysin are naturally occurring materials such as glucose and proteins. The effect of rabbit serum and of glucose on hemolysis by β-lysin is shown in Table II. It will be seen that hemolysis is increased in the presence of low concentrations of serum, whereas higher concentrations cause a marked inhibition of hemolysis by β-lysin. Glucose exhibits a slight inhibitary effect.

TABLE II

EFFECT OF GLUCOSE AND RABBIT SERUM ON HEMOLYSIS BY β-LYSIN

Material added	Activity, units/ml
Control	2260 2645
Glucose, 0.16%	2270 2290
Rabbit serum (1:700)	2940 2940
Rabbit serum (1:70)	4200 4200
Rabbit serum (1:28)	2310 2270
Rabbit serum (1:14)	1300 1220

In order to determine whether the apparent activation by serum was due to proteins, gelatin and crystallized bovine serum albumin (Armour) were added in varying concentrations to the test system. The proteins were dissolved in the usual phosphate–saline buffer containing 0.010 *M* magnesium sulphate and readjusted to pH 7.0 by addition of *N* sodium hydroxide. The indicated levels of protein were obtained by addition of the required volume of protein solution to the usual test system. The data obtained (Table III) show that both proteins have an activating effect on hemolysis by β-lysin. Maximum activation occurs with 1 to 1.5% gelatin and with 1.5 to 2.5% albumin. The latter produced a consistently greater enhancing effect than gelatin.

TABLE III

EFFECT OF PROTEINS ON HEMOLYSIS BY β-LYSIN

Concn. protein, %	Hemolytic activity, units/ml in presence of:	
	Gelatin	Albumin
0.0	1022 840	1022 994
0.1	1680 1470	
0.5	2350 2310	3080 3020
1.0	2520 2480	
1.5	2580 —	3545 3530
2.0	2360 2420	
2.5	2280 —	3500 3500
3.5		3530 3820

NOTE: Gelatin and albumin dissolved in usual saline–phosphate buffer containing 0.015 *M* $MgSO_4$ and readjusted to pH 7.0 by addition of *N* NaOH. Concentration of gelatin and albumin 3.5 and 7% respectively.

As gelatin has been employed to protect toxins against spontaneous loss of activity in dilute solution (8), it was thought that the effects of protein on hemolytic activity noted above may have been due to a similar action. That this was not the case in the present instance was shown by the following experiment. When a number of tubes containing dilute lysin in buffer were allowed to incubate at 37° C for 3 to 25 minutes before addition of erythrocytes for completion of the test, the resulting hemolysis showed no significant reduction as a consequence of the prolonged incubation period before addition of erythrocytes. Hence addition of proteins does not cause increased activity by protecting β-lysin against spontaneous loss of activity.

An inexplicable and sometimes extreme variation in hemolysis between duplicate tubes was often encountered during the course of this study. It was noted, however, that variation was much reduced in the presence of the optimum concentrations of protein employed in these tests. For this reason gelatin is routinely incorporated in the buffers at a concentration of 1%, and the pH readjusted to 7.0 by addition of *N* sodium hydroxide.

Sensitivity to β-Lysin of Erythrocytes from Various Species

It has been reported previously (2, 3, 11) that bovine and sheep erythrocytes are relatively susceptible and rabbit erythrocytes resistant to hemolysis by β-lysin. Erythrocytes of human origin have been reported (2, 9, 11) to be slightly susceptible to hemolysis by this lysin. It was thought necessary, however, to determine the relative sensitivity of erythrocytes from various sources employing the partial hemolysis method and buffers containing magnesium ions. The relative hemolytic activity of β-lysin toward erythrocytes from sheep, rabbits, and man are shown in Table IV. Erythrocytes from sheep were the most susceptible, from rabbits essentially resistant, and from humans slightly susceptible to the lytic action of this lysin. A concentration of lysin approximately 120 times that required for hemolysis of sheep erythrocytes is required to cause comparable lysis of human erythrocytes.

TABLE IV

SENSITIVITY OF VARIOUS ERYTHROCYTES TO β-LYSIN

Species	Dilution of β-lysin	% hemolysis*	
		Hot	Cold
Sheep	1:600	10	80
Rabbit	Undiluted	6	23
Rabbit	1:10	Nil	11
Human	1:5	5	64
Human	1:10	3	45

*Average of duplicates.

Effect of Erythrocyte Concentration on Hemolysis

Since erythrocytes serve as substrate in the system under study, it is necessary to determine the effect of changes in erythrocyte concentration upon extent of hemolysis obtained with a given concentration of lysin. A series of duplicate tubes were prepared containing various quantities of erythrocyte suspension, 1 ml of diluted lysin, and the necessary volume of buffer to give a total of 7 ml; all components were suspended or diluted in the usual saline–phosphate buffer, pH 7.0, containing 0.01 *M* magnesium sulphate. Lysin and buffer were added to the tubes which were then incubated for 5 minutes at 37° C prior to addition of the indicated volume of erythrocyte suspension (Table V). The effect of variations in erythrocyte concentration on the extent of hemolysis is quite marked, and hence, this variable, if not controlled, may be a source of error in the estimation of lysin activity by this method.

TABLE V

EFFECT OF ERYTHROCYTE CONCENTRATION ON EXTENT OF HEMOLYSIS CAUSED BY β-LYSIN

Ml R.B.C. suspension	Complete hemolysis, D_{541}	"Hot–cold" lysis, D_{541}	% hemolysis
2.0	0.715	0.210 0.200	28.7
1.5	0.580	0.205 0.203	35.2
1.0	0.410	0.205 0.198	49.2
0.75	0.305	0.190 0.175	59.7
0.50	0.218	0.160 0.163	73.8
0.25	0.118	0.112 0.103	91.1

Discussion

An examination of "hot–cold" hemolysis caused by β-lysin revealed that the effectiveness of the lysin may be influenced by a number of factors, some hitherto unrecognized. The most striking of these is the marked activating effect of magnesium and manganese ions. For although Walbum (12) noted that addition of a number of divalent ions, including magnesium and manganese, caused "staphylolysin", later (5) considered to have been β-lysin, to show some increase in hemolytic activity, the observed increases were of a small order of magnitude and, as far as the present authors are aware, were not studied or recognized in subsequent work. This activating effect would be particularly significant in studies where the crude lysin may be dialyzed or precipitated before a hemolytic activity test is performed. Moreover, crude lysin, released into a growth medium which may contain adequate magnesium for activation of the lysin, would be so reduced in concentration during the usual dilution procedure for determination of hemolytic activity that the observed end point could in reality mark the point at which magnesium concentration became the limiting factor. It appears most probable that the discrepancy noted by Elek and Levy (4) between plate and tube tests for β-lysin activity is due to failure to provide an adequate concentration of magnesium ions in the dilution or tube tests.

In addition the hemolytic activity of β-lysin is strongly influenced by pH. At reactions slightly below neutrality, hemolytic action is drastically reduced. It is apparent that tests conducted under these conditions will give falsely low results. The pH activity curve falls off less sharply on the alkaline side of neutrality, but the optimum pH level is not wide, and tests should be conducted close to neutrality in order to obtain maximum hemolytic activity of β-lysin samples.

Tests for "hot–cold" lytic activity have usually been conducted (10, 11) by incubating the mixture of erythrocytes and serially diluted lysin for 1 hour at 37° C followed by "overnight" storage in the cold, at which time extent of lysin of the erythrocytes is recorded. A closer examination of the time factor in both the "hot" and "cold" phases of this test indicates that reproducible results may be obtained employing a "hot" period of 25 minutes followed by a cooling period of 15 minutes. These results appear to be in disagreement with those of Bryce and Rountree (2), who stated that a cooling period of at least 2.5 hours was required to obtain reproducible data. The discrepancy may be due, however, to the differing end points employed in these experiments. In the present studies results are expressed in terms of degree of partial hemolysis, whereas Bryce and Rountree (2) recorded the dilution of lysin which caused complete lysis of erythrocytes.

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CONTINUOUS CULTIVATION AND SUSCEPTIBILITY TO BOVINE VIRUSES OF CELL LINES DERIVED FROM BOVINE EMBRYONIC TISSUES[1]

ANDREW S. GREIG

Abstract

Two cell lines, one derived from bovine embryo kidney, the other from bovine embryo skin, have been established in continuous in vitro culture. The cells of each line are predominantly of epithelial type in young cultures and become more spindle-shaped in older cultures. The kidney cells have progressed through 45 passages without change in morphology or growth habits, but the skin cells experienced a period of growth recession between the third and seventh passages, followed by the emergence of a new rapidly growing cell type. Each line of cells was shown to support the growth of the virus of infectious bovine rhinotracheitis and the agent of coital exanthema of cattle. In each case virus growth was accompanied by degenerative changes leading to death of the culture cells.

Introduction

Subsequent to the work of Swim and Parker (7) and Chang (2) on the subcultivation of cells of human origin, several papers have appeared dealing with methods of establishing cell lines in continuous cultivation. Although most of these reports refer to human tissue cells, a number involve work on cells from animal tissues. Westwood, Macpherson, and Titmuss (9) and Westwood and Titmuss (10) described work on the establishment of cell lines from tissues of rabbits, monkeys, and man, and drew particular attention to a type of cellular transformation which occurred in several of their culture strains. De Brion (1) reported success in the isolation and maintenance of a line of epithelial cells from calf kidney, and described changes in growth habits in a substrain derived from the original line of cells. Warren and Cutchins (8) successfully passaged in continuous culture cells from bovine lung, muscle, and kidney origin, and tested them for susceptibility to a number of human and animal viruses. A transmissible swine kidney cell was used by McClain and Hackett (5) in studies with vesicular exanthema virus.

The present report deals with the continuous cultivation in vitro of two cell lines, one derived from bovine embryo kidney (BEK), the other from bovine skin (BES), and their subsequent use for the propagation of bovine viruses.

Materials and Methods

Medium

Initial trials showed that both skin and kidney cells would grow in a variety of media, especially in the presence of a high percentage of bovine serum. The medium finally selected provided rapid growth of cells and sustained maintenance properties. It consisted of 0.5% lactalbumin hydrolyzate (enzymatic), 0.5% Bacto tryptose, 0.001% cysteine HCl, and 0.005% phenol

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red indicator in Earle's balanced salt solution. Twenty per cent by volume of adult bovine serum was furnished as supplement. During virus cultivation trials, solution M199 (Difco) of Morgan, Morton, and Parker (6) was used as a maintenance medium without serum. Penicillin, streptomycin, and mycostatin were added when needed at levels not exceeding 500 units or μg of each per ml of inoculum.

Source of Tissue

The skin and kidney tissue sources were obtained from different embryos. In each case, a 3- to 4-month-old foetus was obtained and the appropriate tissues removed and processed within 2 hours.

Treatment of Tissue

The tissues were subjected to a modification of the trypsinization procedure of Dulbecco and Vogt (3) and Youngner (12). Portions of tissue were finely chopped with scissors and placed in a flask containing about 100 times their volume of 0.25% trypsin solution (Difco 1:250) prepared in phosphate-buffered salt solution. The mixture was agitated at room temperature for 1 hour by a magnetic stirring machine. The trypsin solution with its harvest of cells and cell clumps was then decanted into a cold centrifuge bottle and an equal volume of fresh trypsin solution added to the remaining tissue fragments. After further agitation for 1 hour, the second cell harvest was decanted and added to the first. The harvested cells were centrifuged at 200 r.p.m. for 30 minutes, the supernatant fluid discarded, and the sedimented cells resuspended in growth medium. After removal of larger tissue fragments by filtration through a gauze pad, the cell suspension was adjusted to contain about 1,000,000 cells per milliliter and distributed to flasks. Incubation of the flasks was in the stationary position at a temperature of 37° C. Progress of growth was followed by examination of the cells under the low power objective of a microscope. Cell sheets were stained in the flasks by the Giemsa method for photographing.

Subculture of Cells

To harvest cells for subcultivation after sufficient growth had taken place, the growth medium was removed and the cell sheet broken up by adding a quantity of warm (37° C) trypsin solution and incubating the flask at 37° C in a water bath. When the sheet had loosened and fallen away from the glass, the cells were packed in a conical tube by centrifugation at 1000 r.p.m. for 1 minute, resuspended in growth medium, and redistributed to new flasks. Stock cultures were maintained in Roux flasks. T-series (Kontes) flasks were used during virus-inoculation trials.

Inoculation with Virus

The growth medium was poured off the required number of T-flasks and replaced with an equal quantity of maintenance medium containing a dilution of the virus-infected material. Inoculated flasks were examined daily for signs of cytopathic changes.

Results

Kidney Cultures (BEK Line)

From the original embryonic kidney cortex, cultures were prepared in six Roux flasks. Each of the flasks showed evidence of cellular growth within 48 hours, and confluent sheets of cells were obtained after 6 days' incubation. The cells were harvested by trypsinization on the 6th day and were transferred into six new flasks. Firm adhesion of the cells to the glass of the new flasks was evident within 4 hours. Confluent growth was obtained by 3 days and a second repassage made to six flasks. Repassages were made at 3- to 4-day intervals for the next four passages with no attempt to increase the number of flasks.

At the seventh passage, the harvested cells from two flasks were resuspended in twice their original volume of growth medium and distributed to four flasks. Within 4 days confluent sheets of cells had grown in each flask. In all passages since the seventh, each harvested culture has been made to yield two to four transfer cultures.

At the present 45th passage level, the cells of the BEK line appear morphologically the same and have the same growth habits as the cells of the initial passages. The cells, shown in Fig. 1, seem to be predominantly of the epithelial type. In young cultures they have round or slightly oval nuclei and abundant, usually clear cytoplasm. They lie evenly distributed in monolayer sheets with a slightly whorled pattern. In cultures older than 8 or 10 days the cells become closely packed, elongated, and assume more of a spindle-shape. Although passages are usually made at intervals of 4 to 7 days, it has been found that cultures will remain alive for at least 3 weeks without a change of medium, and much longer if the medium is changed at weekly intervals.

Skin Cultures (BES Line)

Skin from the flank of a 3-month-old foetus was used to initiate the BES line of cells. The harvest of trypsinized cells from about three grams of skin was seeded into six T-60 flasks. After 6 days' incubation, the cell sheets formed were harvested and redistributed to another set of six T-60 flasks. Two additional transfers were made at intervals of 5 days without difficulty, and in each case without attempting to increase the number of flasks. It was soon obvious that the cells in the third passage flasks, although they initially adhered well to the glass, were not propagating with their usual vigor. By the 4th day of incubation most of the cells appeared enlarged, irregular in shape, and were developing a pronounced granularity of the cytoplasm. On the 8th day most cells were greatly enlarged, opaque, irregular, and lying distinctly separated from each other. With difficulty a harvest was made, and because the number of cells was greatly reduced, only two T-60 flasks were seeded for passage. During the ensuing 2 months a slow propagation of cells took place which permitted four additional passages. Many of the cells which developed during this period were long and spindle-shaped. In the seventh passage cellular growth proceeded slowly

for about a week and then appeared to cease entirely. After 3 weeks of apparent quiescence, activity again appeared in one of the flasks. This time there was a rapid, multicentric development of a type of cell not previously observed in the cultures. Within another 6 days the surface of the T-60 flask was covered with new cells. These were harvested and distributed to two T-60 flasks where propagation proceeded rapidly to form complete cell sheets within 4 days. Subsequent passages up to the present 25th have progressed without further difficulty.

The new BES cells are morphologically similar to the cells of the kidney strain, but are distinctly different from the original skin culture cells. The latter were variable in size and shape and included triangular, spindle, and polyhedral types. The new cells, shown in Fig. 2, are epithelial in type in young cultures, becoming more spindle-shaped in cultures 10 days old or older.

Virus Cultivation

Two bovine disease agents, the virus of infectious bovine rhinotracheitis and the agent of coital exanthema of cattle, have been used. The rhinotracheitis virus obtained had been adapted to tissue culture (11), and when inoculated into cultures of either BES or BEK cells produced cytopathogenic effects leading to complete cell destruction by 48 hours. A total of seven serial transfers of the virus were made in the BEK line and six transfers in the BES line of cells. A virus titration performed on the infected fluids from the final transfer of each line gave an end-point dilution of between 10^{-6} and 10^{-7} in each case.

The agent of coital exanthema was isolated from vaginal discharge from an infected animal and was found to grow in both BES and BEK cell lines (4). In each line of cells virus growth resulted in destruction of the cells and liberation of an increased concentration of virus. It was found that cellular destruction proceeded more quickly in the BES line than in the BEK line of cells. In the BES line, virus harvested from the 10th tissue culture passage, representing a final dilution of original material of 10^{-28}, was found capable of reproducing coital exanthema in virgin heifers.

Discussion

A finding of considerable interest in the course of these attempts to establish cells in continuous culture was the difference in manner of adaption to in vitro growth shown by the two cell lines. The BEK cells have presented no difficulties or unusual features throughout the progression of subcultures, and the cells of the 45th passage appear the same as those of the initial passage, both morphologically and in growth rates. The BES cultures, on the other hand, experienced such a marked period of retrogression between the third and seventh passages that it appeared for a time that a stable cell line would not be established. There was, however, a remarkable reappearance of cellular activity and new cell growth in a flask of the seventh passage which

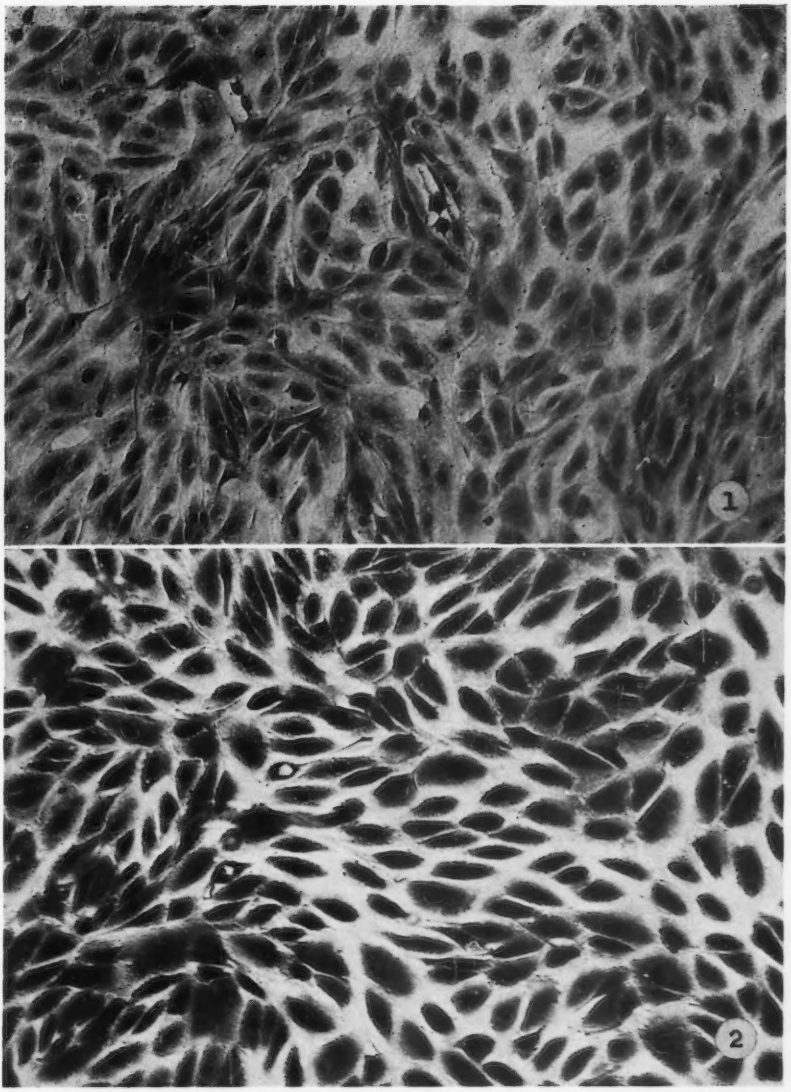

FIG. 1. Bovine embryo kidney (BEK) culture, passage 40, 5 days' incubation. Fixed with 10% formalin, stained with Giemsa. × 500

FIG. 2. Bovine embryo skin (BES) culture, passage 19, 4 days' incubation. Fixed with methyl alcohol and stained with Giemsa. × 500

followed a period of quiescence and apparent cellular degeneration. This reappearance of cellular activity was accomplished by a type of cell morphologically different from those of the original cultures. The new cells were more uniform, more like epithelial cells, and multiplied with greater rapidity than the cells in the original cultures. The whole process would suggest that some fundamental change had taken place in the cells involved during the period of retrogression, either similar to or identical with the cellular transformation described by Westwood, Macpherson, and Titmuss (9).

These workers observed that fibroblasts were always present prior to transformation, that transformation occurred in each case when cellular multiplication was slow or had ceased, and that the appearance of new cells in a culture was multicentric. They concluded that transformation was not induced by any specific modes of handling, in either preliminary handling or subsequent treatment of the cultures. In another paper Westwood and Titmuss (10) advanced reasons to show that transformation was related to factors other than simple mutation.

The marked recession in an early passage culture, the sudden, multicentric reappearance of cell growth, and the change in morphology and growth habits of the new cells would suggest that the BES line is composed of transformed cells. The lack of any such changes in the BEK line would indicate that the kidney cells did not undergo transformation.

The initial purpose underlying the attempts to establish cell lines of bovine origin in continuous culture was to provide practical, reliable, and stable cells for the growth of bovine viruses. This purpose appears to be at least partially fulfilled by the successful cultivation of the viruses of infectious bovine rhinotracheitis and coital exanthema. Whether or not the cell lines will remain stable indefinitely cannot be predicted. Attempts are being made to preserve the cell lines in long-term storage by freezing. Further trials are in progress to cultivate other bovine viruses and viruses of other species in each of the cell lines.

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A BACTERIOPHAGE THAT ATTACKS NUMEROUS PHYTOPATHOGENIC XANTHOMONAS SPECIES[1]

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Abstract

This paper reports the isolation of a lytic phage that attacks in vitro numerous phytopathogenic *Xanthomonas* species, including *X. campestris* (Pammel) Dowson, the cause of black rot disease of crucifers. Although 'one-step' growth experiments suggested an average burst size of ca. four for this phage–host system, 'single burst' experiments indicated a burst size of ca. one hundred phage particles per bacterium. The particles have typical phage morphology, as determined by electron microscopy. This phage gave satisfactory results when used in the rapid plaque count test for detection of phage-sensitive bacteria in plant materials.

Introduction

Bacteriophage (bacterial virus) particles can multiply only within susceptible host bacteria and in doing so cause lysis of the bacterial cells. An increase in the numbers of phage particles present in a given suspension is, therefore, evidence of the presence of host bacteria within that suspension. Susceptibility of certain phytopathogenic bacteria to lysis by specific phages was employed by Katznelson and Sutton (5, 9) and by Katznelson, Sutton, and Bayley (7) as the basis of a sensitive method (the rapid plaque count method) for the detection of seed-borne bacterial plant pathogens. They incubated macerated plant materials with phage of known specificity; an increase in the number of phage particles during incubation indicated the presence of host bacteria in the plant material. Thomas (10) and Thornberry *et al.* (11) have also used susceptibility to specific phages in the identification of some phytopathogenic bacteria.

The susceptibility or resistance of bacterial strains to lysis by reference strains of phage is frequently used as a character in the epidemiological tracing of bacterial pathogens (1). The significance for bacterial taxonomy of patterns of resistance or sensitivity to various phages has been discussed by Stocker (8). It is sufficient to say here that bacterial strains which have in common a sensitivity to a given phage are presumed to be closely related. To be generally useful, the rapid plaque count method (5, 7, 9) for the detection of host bacteria requires that the reference phages employed attack and lyse a wide range of strains within a bacterial species or genus. Experience with phages for *Xanthomonas translucens* (6) suggested that the isolation of species-specific phages attacking all strains of a species was unlikely. This paper reports the isolation and characterization of a phage (Pg 125) that attacks a wide range of *Xanthomonas* species including the economically important *X. campestris*, causal organism of black rot of crucifers.

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Methods

Isolation

The polyvirulent *Xanthomonas* phage Pg125 was isolated from a suspension of macerated swede seed, compost, and sewage, in nutrient broth heavily inoculated with *X. campestris*. After 48 hours at room temperature 10-ml aliquots of the suspension were centrifuged and the supernatant then filtered through sintered glass to remove bacteria. Aliquots of this bacteria-free filtrate were added to a broth culture of *X. campestris*, incubated 24 hours at 27° C, and the bacterial cells then removed by filtration. Three serial passages of the phage were made in this way before visible lysis occurred in broth cultures. Single plaque isolations were made prior to testing the host specificity of this phage.

Lysates

High titer phage lysates were obtained by lysis of broth cultures (titer ca. 2.5×10^9 particles/ml) or by the soft agar layer method of Hershey *et al.* (4), by which phage titers of ca. 10^{10}/ml were obtained. Phage titers were determined by the soft agar layer method of Adams (2).

Determinations of Phage Sensitivity of Bacteria

Bacteria for use in experiments were grown on yeast beef agar slants. To test for phage sensitivity about 5×10^8 growing bacteria and 5×10^9 phage particles were mixed in a final volume of 12 ml nutrient broth contained in a 50-ml Erlenmeyer flask, and incubated at room temperature for 24 hours. Cultures were then examined for visible clearing and compared with cultures grown in similar control flasks without added phage.

Characteristics of the Phage

'One-step' growth studies of the multiplication of phage Pg125 in host strain (P125) of *X. campestris* were made by the methods of Delbrück and Luria (3). Electron micrographs were made of chromium-shadowed collodion pseudoreplicas (12) of agar plates on which phage lysis of *X. campestris* had occurred.

Bacterial Cultures

The bacterial strains used were from the stock culture collection of the Botany and Plant Pathology Laboratory, Science Service, Canada Department of Agriculture, Ottawa.

Results and Discussion

General

Phage Pg125 when plated on *X. campestris* by the soft agar layer method produced small (ca. 1 mm) clear plaques of varying diameter after 24 hours' incubation at 27° C. At temperatures below 27° C the plaques were less clear and the apparent titer of lysates was reduced. Above 27° C the small plaques were masked by rapid growth of the host. The efficiency of plating was also reduced by previous drying of the basal layer of nutrient agar.

PLATE I

FIG. 1. Electron micrograph of phage particles of Pg125. Chromium-shadowed collodion pseudoreplicas of agar surface on which lysis of infected bacteria had occurred. Magnification, ×43,000.

To determine the characteristics of the multiplication of this phage in bacteria of an *X. campestris* strain (P125), one-step growth experiments were carried out. Preliminary experiments showed that absorption of the phage particles by the bacteria was slow. Consequently a period of 30 minutes was allowed before samples of the mixture of phage and bacteria were diluted to prevent further absorption or centrifuged to determine the proportion of phage particles absorbed. In one-step experiments diluted samples of the mixture of phage and bacteria were plated in a soft agar layer at 15-minute intervals for a period of 5 hours. Plaques appearing on the plates were counted after 24 hours' incubation at 27° C. In one experiment the absorption mixture contained 5.7×10^8 bacteria of strain P125 and 3.1×10^8 phage particles/ml. Absorption in the host cell/phage mixture after 30 minutes was about 40% and the average number of phage particles absorbed per bacterium was 0.23. The 'latent' period was 210 minutes followed by a 'rise' period of 60 minutes during which newly formed phage particles were liberated by lysis of infected bacteria. An average burst size of 4.6 phage particles per infected bacterium was calculated; in other experiments values of ca. four and ca. twelve were found. The mean generation time of the host in shake culture was about two hours.

'Single burst' experiments were carried out in which phage infected cultures of P125 were diluted and plated so that an average of 0.25 infected bacteria (calculated by the Poisson formula from the proportion of plates which received *no* infected bacteria) were present on the nutrient agar surface in each of a series of petri dishes. After 6 hours from the time of phage infection the plates were flooded with a soft agar layer containing phage-sensitive indicator bacteria, in order to detect as plaques any phage particles which had been liberated by the lysis of the infected bacterium originally plated. The results indicated that up to ca. one hundred phage particles could be liberated by an infected bacterium. The discrepancy between the low average values obtained for burst size and higher individual values would be accounted for if only a proportion, rather than the whole, of the infected bacteria burst during the initial 'rise' period.

Electron micrographs showed Pg125 phage particles to be sperm-shaped with heads of about 60 mμ in diameter and tails of about 180 mμ in length and 10–15 mμ in width (Fig.1).

Strains Attacked

Fifty-two strains from 25 phytopathogenic species of *Xanthomonas* were tested for lysis in broth by this phage (Table I). Twelve of these strains, including representatives of three species, i.e. *X. beticola*, *X. maculafoliagardiniae*, and *X. rubrilineans* (one strain of each), were not attacked. Lysis was not obtained in broth cultures, nor were plaques produced in layer plates, seeded with phytopathogenic bacteria belonging to other genera, e. g. *Corynebacterium* (two species), *Erwinia* (two species), and *Pseudomonas* (five species).

Practical Application and Conclusions

The presence of susceptible host bacteria in *Pelargonium* cuttings, suspected of infection with *X. pelargonii*, was demonstrated by the technique of Katznelson and Sutton (5, 9). *X. pelargonii* was later isolated from this material and its identity confirmed by plant inoculation tests. Similarly, the presence of *X. campestris* was detected, and later confirmed, in samples of vegetative parts and seeds of various crucifers e.g. turnip, cabbage, and swede.

It is concluded that the polyvirulent phage described in this paper can be used to test for the presence of a wide range of *Xanthomonas* species in plant materials. It should be noted that positive results obtained by the rapid plaque count method do not in themselves establish the presence of a particular pathogen. Ideally, isolation and tests for pathogenicity, as done here for *X. pelargonii* and *X. campestris*, should also be carried out.

TABLE I

Xanthomonas STRAINS TESTED FOR SENSITIVITY TO LYSIS IN BROTH BY PHAGE Pg125

Species	Strain No.
X. begoniae	P-14
X. beticola	P-23*
X. campestris	P-32, P-58, P-125
X. carotae	P-89,* P-124
X. corylina	P-122
X. cucurbitae	P-16
X. juglandis	P-121
X. maculafoliagardiniae	P-90*
X. malvacearum	P-18
X. pelargonii	P-123
X. phaseoli	P-4, P-21, P-57, XP-3, XP-8
X. phaseoli var. *fuscans*	P-5
X. phaseoli var. *sojense*	P-20
X. phleipratense	P-35
X. pruni	P-15, P-29,* P-120
X. rubrilineans	P-107*
X. translucens f. sp. ?	P-84, P-136*
X. translucens f. sp. *cerealis*	P-25, P-37, P-67, P-79
X. translucens f. sp. *hordei*	P-36, P-70,* P-81,* P-82, P-83
X. translucens f. sp. *hordei-avenae*	P-50, P-63, P-64
X. translucens f. sp. *phleipratensis*	P-76
X. translucens f. sp. *secalis*	P-38,* P-68,* P-69
X. translucens f. sp. *undulosa*	P-34, P-65, P-66, P-77, P-78
X. translucens f. sp. *undulosa* or *cerealis*	P-80
X. vesicatoria	P-1,* P-22, P-126*

*Resistant to lysis by Pg125.

Acknowledgments

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A COMPARISON OF SOME PROPERTIES OF STRAINS OF PSEUDOMONAS AERUGINOSA SENSITIVE AND RESISTANT TO QUATERNARY AMMONIUM COMPOUNDS[1]

D. R. MacGregor[2] and P. R. Elliker

Abstract

Pseudomonas aeruginosa was adapted to grow on tryptone – glucose – yeast extract agar containing 2000 p.p.m. alkyldimethylethylbenzyl ammonium chloride (QAC-A). In germicidal trials these resistant cells could be made QAC-A sensitive by treatment with ethylenediaminetetraacetate. In Warburg studies lyophilized preparations of resistant strains were more sensitive to inhibition of substrate oxidation by QAC-A than the normal strain.

It is postulated that quaternary ammonium germicide resistant strains of *P. aeruginosa* owe their resistance to their impermeability to QAC.

Introduction

The ability of some species of bacteria to develop resistance to quaternary ammonium compounds (QAC) is of interest from the standpoint of mechanism of action of this class of bactericides. Chaplin (2) observed that *Escherichia coli* could acquire the ability to grow in media containing 217 p.p.m. alkyldimethylbenzyl ammonium chloride (ADBAC). However, *Micrococcus pyogenes* var. *aureus* did not increase its tolerance to ADBAC on cultivation in presence of the bactericide. In a subsequent report Chaplin (3) developed a strain of *Serratia marcescens* capable of growing in the presence of 100,000 p.p.m. of ADBAC. The resistance of this organism to QAC was postulated to be due to a lipid or lipid–protein material on the cell surface which resisted the disruptive surface force of the disinfectant. Supporting evidence for this was intense staining by Sudan black B, increase in the ether–alcohol-soluble fraction, decrease in electrophoretic mobility, and the removal of resistance by the action of lipase.

The present report deals with investigations designed to elucidate the site of resistance to QAC in *Pseudomonas aeruginosa*. Two approaches were used. First the germicidal effect of QAC against sensitive and resistant strains of this organism, in the presence and absence of a chelating agent, was studied. Secondly, the effect of QAC on the oxidative behavior of resting and lyophilized resistant and nonresistant cells was observed. Chelating agents, and particularly ethylenediaminetetraacetate (EDTA), have been used to effect the release of cell material from *Candida albicans* (4). If *P. aeruginosa* acquires resistance to QAC by becoming impermeable to these compounds, then treatment with a material such as EDTA might, by modifying the cell wall, render resistant cells QAC sensitive. Thus, the inhibition of oxygen consumption of a cell preparation by QAC can also be used as a criterion of its effect on the

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cell. Comparison of resting cells and lyophilized preparations, where permeability is presumably removed, should therefore reveal if development of QAC resistance is due to permeability alteration in the cell wall, or to changes inside the cell.

Methods

Two QAC resistant strains of *P. aeruginosa* were developed. The first (strain Ra) was obtained by inoculating the sensitive organism into tryptone – glucose – yeast extract (TGY) broth containing various levels of alkyldimethylethylbenzyl ammonium chloride (QAC-A). Transfer was made from the highest QAC-A concentration showing growth into another series of TGY – QAC-A broth tubes. The organism acquired a tolerance to 700 p.p.m. of the germicide in nine transfers. Because growth occurred only on the surface of the TGY – QAC-A broth, further subcultures were made on TGY – QAC-A agar to provide more satisfactory growth conditions. The organism was able to grow in the presence of 2000 p.p.m. QAC-A after four further transfers on the solid medium. The pigment-producing property of this organism was lost on repeated transfer in QAC-containing medium. A second resistant strain was developed by heavily streaking the sensitive strain on TGY agar containing 1000 p.p.m. QAC-A. Two similar, large, yellow-green pigmented colonies appeared in 4 days. One of these colonies was transferred to TGY agar containing 1500 p.p.m. and growth from this medium was transferred to 2000 p.p.m. QAC-A. The resultant culture continued to produce pigment and was designated strain Rb. Media containing more than 2000 p.p.m. QAC-A gave erratic growth and for this reason no further attempt at increasing the resistance of either strain was made. Both resistant strains were routinely carried on TGY agar slants containing 2000 p.p.m. QAC-A.

Germicidal trials were conducted using the method of Weber and Black (5) with a QAC inactivator at pH 7.2 consisting of 2.222 g of asolectin (lecithin), 15.8 ml of Tween 80, 200 mg $CaCl_2$, and 1.25 ml of 0.25 *M* phosphate buffer per liter. The effect of ethylenediaminetetraacetate (EDTA) on the germicidal activity of QAC-A was tested by combining the chelating agent with the germicide before they were mixed with the cells in the medication tube.

Manometric studies were made using the conventional Warburg techniques for determination of oxygen uptake. Resting cell suspensions were prepared by washing the growth from 24-hour TGY agar bottle slants with 0.067 *M* phosphate buffer. The cells were washed by centrifugation and standardized by the micro-Kjeldahl method to contain 0.3 mg of nitrogen per ml. One milliliter of this suspension was used in each Warburg flask. Final substrate concentration was 0.01 *M* and QAC-A concentration where used was 100 p.p.m. Both substrates and germicide were made up in 0.067 *M* phosphate buffer.

Lyophilized preparations were made by freeze-drying a paste of washed cells under vacuum over phosphorus pentoxide. These dried organisms were used at a level of 20 mg dry weight per flask and were supplemented with cofactors as described by Campbell and Stokes (1). (Substrate concentration was 0.01 *M*, and the QAC-A concentration was 333 p.p.m.)

Results and Discussion

The effect of EDTA on the germicidal activity of QAC-A toward the normal strain (N) and resistant strains Ra and Rb of *P. aeruginosa* is shown in Table I. The controls in which 100 p.p.m. EDTA was tested alone showed some germicidal effect which, however, was not sufficient to mask the results obtained with QAC-A and EDTA used together. In preliminary experiments 250 p.p.m. EDTA alone gave kills as high as 99.999%. This germicidal activity of EDTA may be due to the disruption of the integrity of the cell. The activity of 100 p.p.m. QAC-A with strain N ranged from almost complete to an entire kill between 15 and 300 seconds, while strains Ra and Rb were resistant to the afore-mentioned level of the germicide. However, when QAC-A and EDTA were used together, killing of strains Ra and Rb was of the same order as that obtained with strain N. In view of the possible effect of EDTA upon the cell wall of *P. aeruginosa*, it seems possible that EDTA may accelerate QAC action, at least with QAC resistant strains, by increasing the permeability of the cell to QAC. Conversely it is possible that resistant strains owe their resistance to their impermeability to QAC.

TABLE I

THE EFFECT OF ETHYLENEDIAMINETETRAACETATE (EDTA) ON SENSITIVITY OF N (NORMAL) AND R (RESISTANT) STRAINS OF *P. aeruginosa* TO ALKYLDIMETHYLETHYLBENZYL AMMONIUM CHLORIDE (QAC-A) AT pH 7.4

Strain	Original cell count, $\times 10^6$	QAC-A, p.p.m.	EDTA, p.p.m.	Organisms killed in exposure period of: 15 sec, %	60 sec, %	300 sec, %
N	170	100	None	99.770	99.970	100.000
N	170	None	100	54.000	71.000	57.000
N	170	100	100	99.900	99.999	100.000
Ra	21	100	None	0.0	0.0	0.0
Ra	21	None	100	0.0	84.000	99.500
Ra	21	100	100	99.996	99.998	100.000
Rb	61	100	None	0.0	0.0	0.0
Rb	61	None	100	0.0	0.0	66.000
Rb	61	100	100	99.200	99.800	99.997

The inhibition by QAC-A of glucose and succinate oxidation by normal and resistant *P. aeruginosa* is shown in Table II. Strains Ra and Rb were found to be less sensitive to inhibition by QAC-A than strain N. However, the difference is not as marked as in the germicidal trials. Inhibition of substrate penetration of the cell is a possible explanation for the partial inhibition of oxidation of substrate with the resistant organisms.

TABLE II

INHIBITION BY ALKYLDIMETHYLETHYLBENZYL AMMONIUM CHLORIDE (QAC-A) OF SUCCINATE AND GLUCOSE OXIDATION BY RESTING CELL SUSPENSIONS OF N (NORMAL) AND Ra, Rb (RESISTANT) STRAINS OF *P. aeruginosa*

Substrate, 0.01 *M*	QAC-A, p.p.m.	Oxygen uptake by strains			Inhibition of strains*		
		N, Q_{O_2}(N)	Ra, Q_{O_2}(N)	Rb, Q_{O_2}(N)	N, %	Ra, %	Rb, %
None	None	103	95	170			
None	100	115	215	325			
Glucose	None	1460	815	1018			
Glucose	100	156	450	490	97	67	80
Succinate	None	1160	575	350			
Succinate	100	155	405	360	96	60	80

NOTE: 0.3 mg of bacterial nitrogen per flask.
*Oxidation in absence of substrate subtracted in calculation of per cent inhibition.

TABLE III

INHIBITION BY ALKYLDIMETHYLETHYLBENZYL AMMONIUM CHLORIDE (QAC-A) OF SUCCINATE AND GLUCOSE OXIDATION BY VACUUM-DRIED CELL PREPARATIONS OF STRAINS N (NORMAL) AND Ra, Rb (RESISTANT) STRAINS OF *P. aeruginosa*

Substrate, 0.01 *M*	QAC-A, p.p.m.	Oxygen uptake by strains			Inhibition of strains*		
		N, Q_{O_2}(N)	Ra, Q_{O_2}(N)	Rb, Q_{O_2}(N)	N, %	Ra, %	Rb, %
None	None	162	40	28			
None	333	78	29	23	52	27	18
Glucose	None	287	155	216			
Glucose	333	235	62	88	0	63	65
Succinate	None	281	101	88			
Succinate	333	187	42	28	8	78	92

NOTE: Experimental conditions: 20 mg of dried cells per flask. Nitrogen content per mg: strain N, 0.121 mg; strain A, 0.119 mg; strain B, 0.109 mg. Each flask was supplemented with 1 mg cytochrome *c*, 109 μg of diphosphopyridine nucleotide, and 2 micromoles $MnCl_2.6H_2O$.
*Oxidation rate in absence of substrate subtracted in calculation of per cent inhibition.

The data in Table III show the QAC-A inhibition of glucose and succinate oxidation by lyophilized cells. The endogenous respiration of all three strains is inhibited by QAC with strain N being the most sensitive. However, glucose oxidation by strain N was not inhibited while strains Ra and Rb were inhibited 63 and 65%, respectively. Succinate oxidation by strains N, Ra, and Rb was inhibited 8, 78, and 92%, respectively. Thus it appears that dried preparations of the resistant strains are more sensitive to inhibition of substrate oxidation than the sensitive strain, results which are the reverse of those obtained with resting cells. This relative increase in sensitivity of strains Ra and Rb on lyophilization may be due to removal of the QAC-A permeability barrier of the resistant cells. Two possible explanations for the greater sensitivity of the lyophilized resistant cells as compared to lyophilized normal cells are: (*a*) The resistant cells contain a large amount of inert

material which does not react with QAC, thus giving a higher germicide to active cell material ratio than is revealed by nitrogen or dry weight determinations. (*b*) The normal strain contains more of some nonrate limiting QAC sensitive enzyme or cofactor and therefore shows a lower apparent percentage inhibition during substrate oxidation. Strain N showed greater oxidizing activity than strains Ra and Rb in both the resting cell and lyophilized preparations.

The data indicate that the mechanism of QAC resistance in *P. aeruginosa* may be similar to that found by Chaplin (3) in *S. marcescens*. This QAC obstructing layer is apparently quite permeable to glucose and succinate in the absence of QAC. The effect of EDTA appears similar to that of lipase in disrupting the resistant layer and indicates the possibility of a lipid or lipid–protein complex bound together by multivalent inorganic cations.

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CAPSULAR POLYSACCHARIDES OF ESCHERICHIA COLI, TYPES K28A AND K34A[1]

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Abstract

Examination of *Escherichia coli* isolates from 100 diagnostic stool, urine, or blood cultures yielded 13 mucoid strains, identified by strict biochemical criteria. Specific capsular and agglutination reactions identified four strains as K28A (O group 8), four as K34A (O group 9), and two as K42A, whereas three strains were serologically distinct from each other and from the recorded A types.

Polysaccharides, identified as the respective capsular materials by inhibition of the specific capsular reactions, were isolated from a K28A strain and a K34A strain, grown in a chemically defined medium. The serological individuality of these polysaccharides correlated with a difference in their component monosaccharides, as determined by chromatography and colorimetry. Both contained galactose, glucose, mannose, and from 10 to 14% of a hexuronic acid, but the K28A polysaccharide contained in addition 13% of fucose.

Normal human sera collected in the Rochester area contained antibodies to the K28A and K34A polysaccharides very infrequently and in very low titer. On the other hand, two thirds of such sera contained O group 8 antibody and the other third contained O group 9 antibody, both in much higher titer.

Introduction

Knowledge of the antigenic structure of *Escherichia coli* has culminated in the authoritative monograph of Kauffmann (18), published in 1951. The essential feature of the classification schema is the allocation of *E. coli* antigens to three classes, somatic or O antigens, flagellar or H antigens, and envelope or K antigens. Any organism possessing an envelope antigen exhibits the phenomenon of O inagglutinability, that is, it fails to be agglutinated by homologous O antibodies. In the Kauffmann *Escherichia* schema, strains of *E. coli* are grouped on the basis of their O antigens and typed on the basis of their K antigens. If further differentiation should be desired the flagellar antigens are identified.

Smith *et al.* (30, 31, 32) in 1927 first isolated encapsulated strains of *E. coli* from cases of white scours in calves. The earliest immunochemical work on the capsular substance was reported also in 1927 by Smith (29), who presented evidence of two hexoses and a uronic acid in hydrolyzates of a polysaccharide from one of these strains. Morgan and Beckwith (22), utilizing low incubational temperatures for the production of mucoid growth of *E. coli*, demonstrated type specificity in extracts of the polysaccharides prepared from their strains. Recently, Beiser and Davis (2) reported the presence of galactose, fucose, hexuronic acids, and an unidentified component in hydrolyzates of a polysaccharide from a mucoid strain of *E. coli* whose K type, however, was not identified.

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[2]The data for this publication were taken from a thesis submitted to the University of Rochester in partial fulfillment of the requirements for the degree, Doctor of Philosophy, by Bill B. Wiley. Present address: University of Saskatchewan School of Medicine, Saskatoon, Saskatchewan, Canada.

The present report concerns the distribution of encapsulated strains (KA types) of *E. coli* encountered in diagnostic cultures in this laboratory, the purification and characterization of the capsular polysaccharides of types K28A and K34A, and the occurrence of the corresponding antibodies in the sera of persons in the vicinity.

Materials and Methods

Strains of E. coli

A chance observation revealed that when the medium was kept slightly alkaline at all times certain strains of *E. coli* produced at 37° C a very mucoid growth resembling that of mucoid *Aerobacter* and *Klebsiella* species. Such strains were most conveniently detected on eosin – methylene blue agar plates containing 5% calcium carbonate as a buffer. Cultures were identified as *E. coli* by positive indole, positive methyl red, negative Voges-Proskauer, and negative citrate tests, carried out as described in the ninth edition of *Standard Methods for Examination of Water and Sewage* (1). The identification was confirmed by inability to hydrolyze urea or ferment inositol, properties which Kauffmann (18) has shown to be important for the characterization of *E. coli*.

Medium

The medium employed for preparation of polysaccharide fractions and vaccines was a modified Freeman's medium (13). The basic solution contained potassium dihydrogen phosphate (anhydrous), 3.75 g; ammonium sulphate, 0.42 g; ammonium chloride, 0.42 g; sodium hydroxide, 0.92 g; sodium bicarbonate, 0.84 g; phenol red, 0.01 g; and distilled water, 1000 ml. The solution of these ingredients was adjusted to pH 7.2–7.4 and autoclaved at 121° C for 15 minutes. To each liter of the medium was added aseptically 10 ml of a 4% solution of magnesium sulphate (septahydrate) and 10 ml of glycerol autoclaved separately at 121° C for 15 minutes.

Vaccines and Immunization

For vaccine production, mucoid and O strains of *E. coli* were grown in the synthetic medium for 24 hours at 37° C. The cells were collected by centrifugation and resuspended in sufficient 0.5% formalin in 0.85% sodium chloride solution ("saline") to give a reading of 55 in a Klett–Summerson photoelectric colorimeter with filter No. 42 (approximately 3×10^8 cells per ml). Doses of vaccine (0.2 ml) were injected intravenously into young adult albino rabbits on each of 3 consecutive days at the beginning of each week for a total of 3 weeks. After a week of rest, the animals were bled for antisera.

Serological Procedures

Tests for specific capsular reactions* were done in a conventional manner, using 18- to 24-hour cultures on the synthetic medium solidified with 1.5%

*The term "specific capsular reaction" is used instead of "capsular swelling reaction" in accordance with the evidence summarized by Tomcsik (33).

agar. To demonstrate inhibition of specific capsular reactions by purified polysaccharides, 2 volumes of a 0.2% solution of polysaccharide and 1 volume of antiserum were mixed, held at 37° C for 30 minutes, and centrifuged at 5° C. The supernate was tested for its ability to produce the specific capsular reaction, in comparison with antiserum diluted with 2 volumes of saline. Precipitation tests were done by the conventional layering technique using undiluted antisera and serial decimal dilutions of polysaccharides in saline. For agglutination tests, serial twofold dilutions of antisera in saline were mixed with equal volumes of bacterial suspensions made up in 0.5% formalin in saline to read 100 in a Klett–Summerson photoelectric colorimeter with filter 42. The tests were incubated at 50° C for 18 hours. The end point was considered to be the highest final dilution of antiserum that produced detectable agglutination. For hemagglutination tests, a buffer of the following composition was used as diluent: sodium dihydrogen phosphate (monohydrate), 5.52 g; sodium chloride, 5.85 g; sodium hydroxide, 1.22 g; distilled water, 1000 ml. The pH was adjusted to 7.2 if necessary. To sensitize erythrocytes, solutions of polysaccharides or of O antigens at appropriate concentrations were added to equal volumes of 5% suspensions of washed sheep erythrocytes. After incubation at 37° C for 2 hours, the cells were washed three times by centrifugation and resuspension in the buffer, and finally suspended to a concentration of 1%. Equal volumes of such suspensions and dilutions of antisera were mixed, incubated at 37° C for 2 hours, refrigerated overnight, and examined for hemagglutination.

Preparation of O Antigen

Solutions of O antigens were prepared from type strains of O groups 8 and 9 of the Kauffmann (18) *Escherichia* schema. The organisms were grown in the liquid synthetic medium for 48 hours at 37° C on a shaking machine. The cells were sedimented by centrifugation, washed three times by centrifugation and resuspension in saline, and finally suspended in a small volume of saline. These suspensions were heated in a boiling water bath for 1 hour to extract O antigen. The cells were sedimented and the supernatant fluid was treated with 4 volumes of 95% ethanol. The precipitate of crude O antigen was collected by centrifugation and dried to constant weight over concentrated sulphuric acid *in vacuo*. Finally the crude O antigen was dissolved at a concentration of 0.1% in saline. Landy *et al.* (21) have shown that treatment of *Salmonella typhosa* O antigen with dilute sodium hydroxide at 37° C overnight hydrolyzes the antigenic complex, liberates bound lipids, and greatly increases the affinity of the O antigen for erythrocytes without impairing its immunologic specificity. For this reason, all O antigen preparations used in hemagglutination tests were treated with an equal volume of 0.04 *N* sodium hydroxide at 37° C for 18 hours. The solution was then neutralized and made up to 4 volumes. The resulting 0.025% solution was employed to sensitize sheep erythrocytes.

Analytical Procedures

The nucleic acid content of the polysaccharide preparations was estimated by comparing the absorption curves in the region of 260 mμ of 0.01% aqueous solutions of the polysaccharides with that of a 0.001% aqueous solution of yeast nucleic acid, using a Beckman model DU spectrophotometer.

The glycogen content of the polysaccharides was estimated by the method described by Koch and Hanke (19). This test was found capable of detecting 100 μg but not 10 μg of glycogen.

Nitrogen was determined by a micro-Kjeldahl method.

Total and inorganic phosphorus were determined by a modification of the method of Fiske and Subbarow (17).

Maximum yields of reducing substances were obtained when the polysaccharides were hydrolyzed in 1.0 *N* hydrochloric acid at 100° C for 5 hours. The reducing value was measured as glucose by the method of Schales and Schales (27). Chromatograms of such hydrolyzates were developed on Whatman No. 1 filter paper strips 16.75×1.5 in. The paper was prepared by washing overnight in 0.05 *N* ammonium hydroxide, rinsing three times in distilled water, and drying at 110° C. The hydrolyzates were neutralized with sodium hydroxide and dried over concentrated sulphuric acid *in vacuo*. Pyridine was used to extract the sugars and leave behind the sodium chloride from the hydrolyzates because the presence of large amounts of salts causes the production of dry fronts and smearing of the spots. Each chromatogram was developed by the ascending method from the hydrolyzate of from 1.0 to 1.5 mg of polysaccharide, using *n*-butanol–pyridine–water (3:1:1) (16) for 18 hours at either 37° or 23° C. In order to increase the separation of the spots, at the end of a run the strips were dried at room temperature and replaced in the solvent for an additional run. Usually three or four runs were necessary for good separations. Finally the dried strips were sprayed with 2.5% aniline phthalate in butanol (25). The sprayed strips were dried at room temperature and then heated at 110° C for 20 minutes to develop the color.

The identity of sugars indicated by R_f values was confirmed colorimetrically. The location of the spots was determined by spraying one strip in a run of 10–12 identical strips. The corresponding areas were cut off the remaining strips, eluted into distilled water, and tested by various colorimetric procedures. The carbazole reaction according to Gurin and Hood (14) served quite well in the differentiation of glucose, galactose, and mannose. For methyl pentoses, the technique of Dische and Shettles (8) was used. Since this test does not distinguish between rhamnose and fucose, final identification of these sugars had to be by means of R_f measurement. Attempts to identify the uronic acid components of the hydrolyzates made use of Dische's galacturonic acid test (7) and Dische's glucuronic acid test (6). The general hexuronic acid reaction of Dische (5) was utilized to determine the amount of hexuronic

acid in hydrolyzates of the polysaccharides. Standards of similarly hydrolyzed glucuronic and galacturonic acids were included in the test. The hexosamine content of hydrolyzates of the polysaccharides was determined according to the method of Palmer *et al.* (24).

Results

Examination of more than 100 *E. coli* isolates from diagnostic cultures yielded 13 mucoid strains, 10 from urine cultures, 2 from stool cultures, and 1 from a blood culture. The specific capsular reaction of all these strains with their homologous antisera showed that their K antigens were of the A type. Tests for capsular reactions with the heterologous antisera divided these strains into well-defined groups, which were then identified by the sharply specific capsular reactions between the respective antisera and type strains of all the 16 A types listed by the Kauffmann (18) schema. Four strains were K28A, 4 were K34A, and 2 were K42A, whereas 3 strains were serologically distinct from each other and from the 16 recorded A types. Since types K28A and K34A predominated, one strain of each was selected for the study of capsular material. These strains were shown to belong to O groups 8 and 9, respectively, by specific agglutination of their O variants, isolated as described by Kauffmann (18), with antisera to type strains of these O groups. The 16 A types listed by Kauffmann (18) belonged to either O group 8 or 9.

Two-liter Erlenmeyer flasks containing 1 liter of the synthetic medium were inoculated with 50 ml of an 18- to 24-hour culture in the same medium and agitated at 37° C on a shaking machine during the day. The flasks were left undisturbed during the night to prevent excessive accumulation of fermentation acids. The cultures were maintained for 4 days, although acid production ceased after 60 hours. During growth, the fermentation acids were neutralized with sterile 10% potassium hydroxide at frequent intervals. To the combined contents of those flasks containing only Gram-negative coccobacilli resembling *E. coli*, 4 volumes of 95% ethanol were added. The mixture was refrigerated overnight. The supernatant fluid was then siphoned off and discarded, since it contained little serologically active material. The precipitate of bacterial bodies, nucleoprotein, polysaccharide, and salts from 1 liter of culture, was suspended in 200 ml of distilled water and deproteinized by homogenization in a Waring Blendor with 50 ml of chloroform and 10 ml of *n*-butanol. The emulsion formed by this treatment was centrifuged at 2500 r.p.m. at 5° C and the upper aqueous layer containing the polysaccharide was collected. The precipitate was re-extracted with distilled water to recover any polysaccharide trapped in it. Usually five treatments with chloroform and butanol were necessary before the aqueous layer gave negative biuret and xanthoproteic tests. Finally, 10 g of sodium acetate were dissolved in the protein-free solution and acetic acid was added until the pH was 6.0. Four volumes of 95% ethanol were added and the mixture was refrigerated overnight. The precipitate was collected by centrifugation and dissolved

in 50 ml of 10% sodium acetate whose pH had been adjusted to pH 6.0. This solution was then dialyzed against frequent changes of distilled water in the refrigerator for 4 days. Finally, to the dialyzed solution was added 10 ml of the acetate buffer at pH 6.0 and the polysaccharide was precipitated with 4 volumes of 95% ethanol in the refrigerator overnight. The precipitate was collected by centrifugation and dissolved in the least possible volume of distilled water. The type K28A polysaccharide formed an insoluble lead salt when its solution was treated with aqueous basic lead acetate, made according to the formula in Hawk, Oser, and Summerson (15). The type K34A polysaccharide formed an insoluble copper salt on the addition of aqueous 5% cupric acetate. These insoluble metallic salts of the polysaccharides were collected by centrifugation and the supernatant fluids were tested with more of the precipitants. If no more precipitate formed, the supernatant fluids were discarded. The soluble sodium salts of the polysaccharides were regenerated by dissolving the precipitates in the sodium acetate buffer at pH 6.0 and dialyzing the solutions against distilled water until they gave negative tests for lead or copper respectively (10). Finally, the polysaccharides were precipitated in the presence of the acetate buffer at pH 6.0 by the addition of 4 volumes of 95% ethanol, collected by centrifugation, washed with ethanol, and dried to constant weight *in vacuo* over concentrated sulphuric acid.

The polysaccharides, in dilutions as high as 1:1,000,000, reacted specifically with their homologous antisera. Their relationship to the capsular material of the respective cells was demonstrated by their ability specifically to inhibit the capsular reactions with the homologous antisera.

It seemed likely that the isolated polysaccharides would contain some of the homologous O antigens, for the latter were shown to be present in the supernates of the cultures from which the polysaccharides were prepared, i.e., erythrocytes exposed to these supernates became agglutinable by the homologous O antisera. Accordingly, we estimated the amounts of O antigens present in the purified polysaccharides by their ability to inhibit the agglutination by O antisera of erythrocytes sensitized with the homologous O antigen. Landy *et al.* (21) found that as little as 0.0078 μg of alkali-treated purified O antigen of *Salmonella typhosa* effected detectable inhibition. The results of similar tests employing alkali-treated purified *E. coli* capsular polysaccharides are presented in Tables I and II. Capsular polysaccharide K28A was required in the amount of 1.95 μg to produce the same degree of inhibition as 0.078 μg of *S. typhosa* O antigen did in Landy's experiments, while 0.98 μg of the K34A polysaccharide was required. Landy (20) has presented also evidence indicating a quite uniform degree of serological activity of lipopolysaccharide and polysaccharide antigens from Gram-negative bacilli. Accordingly, we estimate that the K28A polysaccharide contained 4% of *E. coli* O-8 antigen, while the K34A polysaccharide contained 8% of O-9 antigen.

TABLE I

ASSAY, BY INHIBITION OF HEMAGGLUTINATION, OF *E. coli* O-8 ANTIGEN IN PURIFIED K28A CAPSULAR POLYSACCHARIDE

Polysaccharide, μg	Dilution of *E. coli* O-8 antiserum*					
	250	500	1000	2000	3000	Buffered saline
None	+	+	+	+	—	—
0.49	+	+	+	+	—	
0.98	+	+	+	+	—	
1.95	+	+	+	—	—	
3.91	+	+	—	—	—	
7.81	+	—	—	—	—	
15.63	—	—	—	—	—	
31.25	—	—	—	—	—	

*Reciprocal of final dilution before addition of sheep erythrocytes sensitized with alkali-treated O-8 antigen. One-half milliliter of appropriate dilution of alkali-treated K28A polysaccharide was added to 0.5 ml of respective dilutions of antiserum. After the mixtures had incubated for 1 hour at 37° C, 0.5 ml of 1% sheep erythrocytes sensitized with alkali-treated O-8 antigen was added to each tube. After incubation for an additional 2 hours at 37° C and refrigeration overnight, the tubes were examined for hemagglutination. + or — denotes presence or absence of hemagglutination. Unsensitized erythrocytes were not agglutinated by 1:50 dilution of the antiserum.

TABLE II

ASSAY, BY INHIBITION OF HEMAGGLUTINATION, OF *E. coli* O-9 ANTIGEN IN PURIFIED K34A CAPSULAR POLYSACCHARIDE

Polysaccharide, μg	Dilution of *E. coli* O-9 antiserum*					
	250	500	1000	2000	3000	Buffered saline
None	+	+	+	+	—	—
0.49	+	+	+	+	—	
0.98	+	+	+	—	—	
1.95	+	+	—	—	—	
3.91	+	—	—	—	—	
7.81	—	—	—	—	—	
15.63	—	—	—	—	—	
31.25	—	—	—	—	—	

*See footnote to Table I.

Since *E. coli* types K28A and K34A seemed to be the ones most frequently encountered in the Rochester area, it was of interest to determine the incidence of the homologous antibodies in human sera selected at random. Accordingly, the sera of normal individuals which came into the laboratories of the Rochester Health Bureau for routine Wassermann tests were inactivated at 56° C for 30 minutes, diluted in twofold steps from 1:10 to 1:1280, and tested for agglutination of human erythrocytes of blood group O, Rh negative, sensitized with capsular polysaccharide diluted 1:4000 or with alkali-treated O antigen, according to the directions given previously. Of 30 sera tested, only 3 agglutinated the erythrocytes sensitized with K28A polysaccharide, while 1 agglutinated the erythrocytes sensitized with K34A polysaccharide; only one titer (vs. K28A) was higher than 1:40. On the other hand, 15 out of 21 of the same sera agglutinated the erythrocytes

TABLE III

CHEMICAL CHARACTERISTICS OF PURIFIED CAPSULAR POLYSACCHARIDES FROM *E. coli* TYPES K28A AND K34A

Characteristic	K28A polysaccharide, %	K34A polysaccharide, %
Kjeldahl nitrogen	1.11, 1.04	1.04, 1.00
Reducing sugars as glucose	63.4, 60.3	65.0, 62.3
Inorganic phosphorus	0.29, 0.28	0.062, 0.062
Organic phosphorus	0.012, 0.015	0.35, 0.38
Nucleic acid	None	None
Glycogen	<2	<2
Hexosamine	None	2.5

sensitized with O-8 antigen and the other 6 agglutinated the erythrocytes sensitized with O-9 antigen; the titers were much higher, ranging from 1:80 to 1:1280, and were higher in the O-8 group than in the O-9 group.

The general chemical characteristics of the polysaccharides were very similar (Table III). Since nucleic acid was absent, the higher organic phosphorus content of the K34A preparation probably represents phosphorylated carbohydrate or O antigen. The higher inorganic phosphorus content of the K28A preparation probably reflects incomplete dialysis. The relatively high nitrogen contents are not readily explained. They do not derive from nucleic acid or hexosamine and can be only partially accounted for by the amounts of O antigen present. Although both polysaccharides gave negative biuret tests for protein, the nitrogen might represent polypeptide moieties chemically combined to the polysaccharides. If so, the nitrogen values of 1% indicate the presence of approximately six per cent polypeptide.

Chromatograms of hydrolyzates of the polysaccharides, compared with concurrent chromatograms of known sugars and uronic acids, indicated that both contained galactose, glucose, mannose, and a uronic acid as major components, while the K28A polysaccharide contained also a considerable proportion of methyl pentose. These tentative identifications were based on R_f values. They were confirmed by chemical tests of eluates of the respective spots, as described previously. Faint spots giving the characteristic colors of amino acids with ninhydrin also occurred in these chromatograms; they were not analyzed in detail.

The provisional identification of galactose, glucose, and mannose was substantiated by the results of the carbazole test (14), which depends upon differences in the ratio, optical density at 520 mμ ÷ optical density at 420 mμ, of the colors developed. The ratios given by Gurin and Hood (14) are: galactose, 1.10; glucose, 2.65; and mannose, 0.67. We found that after exposure to the conditions of hydrolysis of the present experiments (5 hours, 100° C, 1.0 *N* HCl) galactose and mannose gave ratios of 1.00 and 0.60 respectively, which closely approximate the expected values. However, the same treatment of glucose greatly reduced this ratio. Furthermore, the lower the concentration of glucose, the lower was the ratio. At the concentrations around 100 μg/ml used in these experiments, the treated glucose

gave ratios from 1.38 to 1.61. Three determinations at 150 μg/ml gave an average ratio of 1.54. In comparison, the eluates of the "galactose" spots from the K28A and K34A polysaccharides gave respective ratios of 1.0 and 1.1; the "glucose" spots, 1.48 and 1.52; and the "mannose" spots, 0.67 and 0.65. Comparison of the optical densities of these tests with tests of known amounts of the pure sugars, heated also at 100° C in 1.0 *N* HCl for 5 hours, made possible estimation of the proportions of the three sugars and eventual calculation of the approximate percentages present in the polysaccharides (Table IV).

The "methyl pentose" spot found in chromatograms of hydrolyzates of K28A but not of K34A constituted the principal qualitative difference between the two polysaccharides. Its identity as a methyl pentose was confirmed by the test of Dische and Shettles (8). Since this test does not differentiate fucose from rhamnose, final identification was made by comparing the R_f values of pure fucose (0.41) and rhamnose (0.53), heated at 100° C in 1.0 *N* HCl for 5 hours, with those of the methyl pentose spot (0.41) and material eluted from such spots and rechromatographed (0.41).

Efforts to identify the uronic acid components of the two polysaccharides did not succeed. In our hands, the chromatographic procedure of Fischer and Dörfel (12) did not separate glucuronic and galacturonic acids. The available colorimetric procedures specific for glucuronic and galacturonic acids (6, 7, 9) required separation of the uronic acid fraction from other sugars in amounts not obtainable by the methods of chromatography and elution used in the present experiments. Accordingly, quantitative determinations of the total hexuronic acid contents of hydrolyzates of the polysaccharides were made by the method of Dische (5), using both glucuronic and galacturonic acids as standards. With the former, the hexuronic acid values were one-third higher. Having calculated previously from the results of carbazole tests the proportions of galactose, glucose, and mannose to each other, it was now possible to estimate the fractional distribution of these carbohydrates in the two polysaccharides, using in each case the average of the two hexuronic acid values (Table IV).

TABLE IV

ESTIMATED FRACTIONAL DISTRIBUTION OF CARBOHYDRATE COMPONENTS IN *E. coli* TYPES K28A AND K34A CAPSULAR POLYSACCHARIDES

Component	Type K28A, %	Type K34A, %
Fucose	13.4	None
Uronic acid as:		
Glucuronic	13.2	14.1
Galacturonic	9.9	10.5
Galactose	11	11
Glucose	56	63
Mannose	8	14

NOTE: Fucose and hexuronic acids were determined directly. The proportions of galactose:glucose:mannose were determined by carbazole analyses and the percentages were calculated by difference.

Discussion

Although Smith *et al.* (30, 31, 32) more than 30 years ago clearly correlated encapsulation with virulence of strains of *E. coli* isolated from cases of white scours, a frequently fatal choleriform disease of young calves, the incidence and pathogenic significance of encapsulation in *E. coli* have received comparatively little attention. Vahlne (34) found that 90% of the strains of *E. coli* isolated from infected appendices, 87% of the strains from cases of peritonitis, and 82% of the strains from urinary infections were O-inagglutinable or in other words possessed envelope antigens, as compared with 75% of the strains from normal appendices and 65% of the strains from normal feces. Additional evidence of the importance of encapsulation for the virulence of *E. coli* was provided by Sjöstedt (28), who found that on the average the O-inagglutinable strains were seven times as toxic for mice as the nonencapsulated forms. He also found that the more heavily encapsulated the strain was, the greater was its toxicity, and that phagocytosis of the encapsulated forms occurred only to a slight extent in human blood. The contribution of envelope antigens to invasiveness was substantiated by Sakazaki and Namioka (26), who reported that 70% of the cultures of *E. coli* isolated from internal organs of healthy animals were O-inagglutinable, as compared to only 28% of the cultures from the animals' feces. Finally, it appears that all strains of *E. coli* implicated in infantile diarrhea bear K antigens of the B type (11).

The failure of our limited survey to find in normal human sera a significant incidence of antibodies to the two capsular (A) types of *E. coli* encountered most frequently in our cultural isolations contrasted markedly with the good titers of these sera for the corresponding *E. coli* O antigens 8 and 9. An explanation for this finding could be that the members of O groups 8 and 9 exist mainly in the acapsular phase in the body. Alternatively, the body may not absorb the capsular polysaccharides from the intestinal or urinary tracts and, therefore, no effective antigenic stimulus is provided. It is also possible that the capsular polysaccharides are absorbed, but in a haptenic rather than a fully antigenic state. Finally, the antibodies formed might be of the "incomplete" variety, which would not be detected by the method used.

Smith (29) estimated that hexoses constituted 80% of the polysaccharide she prepared from an encapsulated strain of *E. coli;* uronic acid was present also. Boivin (3) found the capsular polysaccharide of a strain of *E. coli* to be composed of one-third uronic acids and two-thirds neutral sugars, whereas the polysaccharide from another strain contained only neutral sugars. Beiser and Davis (2) identified hexuronic acids, galactose, and fucose in hydrolyzates of their *E. coli* capsular polysaccharide. Unfortunately, the K types of these previous preparations are not known. Fucose, found during the present investigation as a constituent of the K28A polysaccharide, has occurred infrequently in bacterial polysaccharides. Norris *et al.* (23) found it in a polysaccharide from a mucoid variant of *Lactobacillus bifidus;* Wilkinson *et al.*

(35) found it in a polysaccharide from *Aerobacter aerogenes*, type 54; and Davies (4) showed it to be a constituent of somatic polysaccharides from *Salmonella poona*, *Salmonella grumpensis*, and two strains of *Chromobacterium violaceum*.

Studies of capsular antigens must always reckon with contamination by the cognate somatic antigens. Our preparations of the K28A and K34A polysaccharides evidently consisted mainly of the capsular antigens, for they inhibited the respective specific capsular reactions of the parent bacteria with homologous antisera. However, as estimated by a serological method, they may have contained from 4% to 8% of the respective O antigens. An exact determination would require a quantitative precipitin analysis, comparing the reactions of each capsular preparation and the corresponding pure O antigen with homologous O antiserum in the region of antibody excess.

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SIX NEW ESCHERICHIA COLI H ANTIGENS, H41 to H46[1]

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Abstract

Six newly recognized *Escherichia coli* H antigens were characterized as *E. coli* H antigens 41, 42, 43, 44, 45, and 46. These antigens were not significantly related to any previously described *E. coli* H antigens. A new *E. coli* O antigen group (O140) was recorded.

The purpose of the present paper is to characterize six previously unrecognized flagellar (H) antigens occurring in *Escherichia coli* strains. Descriptive literature regarding *E. coli* H antigens 1 to 40 was reviewed by Ewing *et al.* (2), who also outlined methods for the determination of the H antigens of *E. coli* strains. The methods used for the determination of the biochemical and serological reactions of the cultures mentioned herein were the same as those outlined in the above-mentioned publication and were similar to those described by Kauffmann (3), Ørskov (4), and Edwards and Ewing (1).

Available data concerning the O and K antigens and the culture numbers of the strains that are designated herein as standard for the new *E. coli* H antigens are given in Table I, which may be regarded as a continuation of Table XIII in the publication of Ewing *et al.* (2). Culture RVC 1787, O137:K79(L):H41, was isolated by Dr. Rees of the Royal Veterinary College, London, and was received by the writers from Drs. Kauffmann and Ørskov of the International Salmonella and Escherichia Center, Copenhagen. This strain also was the standard O group 137 and K antigen 79(L) culture. Culture P9*c* was received from Dr. Kauffmann in 1949 as the standard O group 70 strain. Through an inadvertent error its H antigen was recorded as H38 by Ewing *et al.* (2). Later it was found that the H antigens of culture P9*c* were not significantly related to H antigens 1 to 41 and accordingly they were designated H42. Cultures 149-51 (O140:K·:H43), 781-55 (O3:K·:H44), and 4106-54, O52*a*,52*b*:K·:H45, were isolated from the stools of infants ill with diarrhea in Mexico, Indiana, and Pennsylvania, respectively. Culture

TABLE I

E. coli STANDARD H ANTIGEN STRAINS

H antigen	O antigen	K antigen	Culture No.
41	137	79(L)	RVC 1787
42	70	·	P9*c*
43	140	·	149-51
44	3	·	781-55
45	52	·	4106-54
46	26	60(B6)	5306-56

NOTE: · = K antigen undetermined.

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TABLE II

E. coli H ANTIGEN RELATIONSHIPS

H antigen	Titer of homologous antiserum	Titer of other H antigens in antiserum		
1	12,800	12; 800*	16; 100*	
2	25,600			
3	12,800			
4	6,400	17; 400		
5	12,800			
6	6,400			
7	12,800			
8	25,600	40; 800		
9	12,800			
10	6,400	9; 200		
11	25,600	21; 400	40; 400	
12	25,600	1; 800		
13	12,800			
14	25,600			
15	25,600			
16	25,600			
17	25,600			
18	12,800			
19	25,600			
20	12,800			
21	25,600	11; 6400		
22	12,800			
23	12,800			
24	25,600	35; 100		
25	25,600			
26	25,600			
27	25,600			
28	25,600			
29	25,600			
30	25,600	32; 3200		
31	25,600	35; 100		
32	12,800	30; 1600		
33	25,600			
34	12,800			
35	12,800			
36	25,600			
37	25,600	41; 100		
38	25,600			
39	25,600			
40	25,600	8; 200	11; 400	37; 200
41	25,600	37; 100	39; 400	
42	12,800	6; 200	26; 100	
43	25,600	37; 100		
44	25,600	39; 400		
45	25,600	20; 100		
46	12,800	17; 800		

NOTE: Blanks indicate no significant relationship. Reciprocal reactions are underlined.
*H antigen 12 reacted to a titer of 1:800 in H1 antiserum and the reaction was reciprocal; H antigen 16 reacted to a titer of 1:100 in H1 antiserum, but the reaction was not reciprocal.

5306-56, O26:K60(B6):H46, was recovered from the feces of a chicken by Dr. Thomson, of the Public Health Laboratory Service, Cardiff, Wales, and was identified among a group of strains sent by Dr. Thomson at the writers' request.

The O antigens of cultures 781-55 and 5306-56 were identical with those of the standard strains of the O groups to which they belonged, i.e. O2 and O26, respectively. However, the O antigens of culture 4106-54 were not identical with those of the standard O group 52 strain. In this instance, reciprocal agglutinin adsorption studies indicated that culture 4106-54 possessed an O antigenic fraction that was not present in the standard O group 52 strain. The O antigens of culture 4106-54 also were related to those of O group 88 strains. The O antigens of the remaining strain (149-51) were related to those of O groups 34, 53, and 85 but none of these relationships was considered extensive enough to warrant classification of culture 149-51 as a member of any of these groups. Hence, a new O antigen group number (140) was assigned to culture 149-51. The antigen designations assigned to the type strains mentioned above, and listed in Table I, were made after consultation with Drs. Kauffmann and Ørskov.

The six newly recognized *E. coli* H antigens were not significantly related to any of the 40 previously described *E. coli* H antigens, nor to each other (Table II). With the exception of a slight reciprocal relationship between H antigens 37 and 41, the minor relationships that were detected were unilateral (Table II). Results obtained to date indicated that antisera prepared with *E. coli* H antigens 41 to 46, inclusive, may be used in diagnostic work at a dilution of 1:1000 without adsorption.

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THE OIL OF WHEAT STEM RUST UREDOSPORES

I. THE STEROL AND CAROTENES OF THE UNSAPONIFIABLE MATTER[1]

F. W. HOUGEN,[2] B. M. CRAIG, AND G. A. LEDINGHAM

Abstract

Uredospores of *Puccinia graminis* var. *tritici*, race 15B, contain approximately 20% by weight of ether-extractable oil which was separated into fatty acid and unsaponifiable fractions. The latter contains a sterol which was isolated and identified as ergost-7-enol. An investigation of the carotenes has confirmed the presence of β- and γ-carotene as the major carotenoid components and further established the presence of the minor components, phytoene, lycopene, a *cis*-β-carotene, and a *cis*-γ-carotene.

Introduction

Conspicuous oil droplets are present in the uredospores of many rusts. Recent studies in this laboratory (10) have shown that in wheat stem rust, the oils constitute nearly 20% of the spore weight, and they are the major endogenous substrate utilized during germination. With the exception of a few studies on the carotenoid pigments, the characteristics and structure of these oils have never been reported. In the autumn of 1955 several hundred grams of uredospores of race 15B *Puccinia graminis* var. *tritici* were collected from a plot of Stewart wheat. Sufficient oil was then extracted from the spores to enable a detailed investigation to be made on both the fatty acid and unsaponifiable fractions. This paper records the characteristics of the sterol and carotenes found in the latter fraction. Studies on the fatty acids will follow shortly.

Newton and Johnson (8) made one of the first investigations of the colored compounds present in normal uredospores as well as in an orange and greyish-brown mutant. They concluded that normal spores and the orange mutant contained carotene but that a further red compound, not extracted by many solvents, was also present in normal spores. Recently, Irvine *et al.* (6) extracted and compared the pigments of the uredospores of five cereal rusts and of flax rust. They reported the presence of β-carotene, γ-carotene, and lycopene in all samples investigated.

Materials and Methods

Uredospores of *Puccinia graminis* var. *tritici*, race 15B, were used in the present investigation. Preliminary chemical investigations were carried out on spores obtained by washing rusted wheat stems and leaves with water. Spores used in the present study were collected by vacuuming the leaves and stems of infected Stewart wheat plants growing under field conditions.

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The spores were sieved through a U.S. 325-mesh screen to remove extraneous material and were stored at +5° C. One sample of fresh spores was supplied by Dr. D. R. Knott, Field Husbandry Department, University of Saskatchewan, and was extracted without delay.

The spores (1- to 20-g batches) were extracted with ethyl ether in a ball mill for 1 to 3 days. The solids were removed by centrifugation and were washed three times with ethyl ether. The solvent was removed and the oil was stored in glass-stoppered flasks under nitrogen at −30° C. The oil was saponified with potassium hydroxide in aqueous ethanol and the unsaponifiable material was extracted with ether from the soap solution. The ether was removed and the unsaponifiable material was stored under nitrogen at −30° C. The fatty acids were recovered by acidification of the soap solution and extraction with ether.

The free fatty acid of the whole oil was determined by the standard A.O.C.S. procedure. The glycerol in the aqueous solution, after removal of unsaponifiable matter and fatty acids, was measured by periodate oxidation according to Lambert and Neish (7).

Chromatographic Procedure

Chromatograms were carried out on columns of alumina of activity II and IV (Brockman's scale) as required. The columns were jacketed and cooled with tap water to prevent excess evaporation of the eluting solvents. The substance to be chromatographed, dissolved in a small volume of the solvent of weakest eluting power used in the chromatograms, was run into the column and eluted as required with light petroleum, light petroleum/ether mixtures, ether, and ether/methanol mixtures, in the order mentioned. In all but a few early chromatograms, a device for gradient elution was used, essentially that of Bock and Ling (3), which gave linear concentration gradients. The solvent systems used are described in the text in an abbreviated form; this is illustrated by the example "gradient 0–20% ether in light petroleum (200 ml)" by which is meant that a continuous and linear increase in ether concentration (0.1% per ml of solvent delivered to the column) was obtained by continuous addition of a solution (100 ml) of 20% (v/v) ether in light petroleum into the mixing reservoir which originally contained light petroleum (100 ml). Successive small fractions of eluate were collected by means of a siphon and examined as required by absorption spectroscopy, weight of solvent-free material, and melting point. Distribution curves of eluted material were made by plotting, against the number of the eluted fractions, either the solvent-free weight (with examinations of unsaponifiable components other than carotenes) or the optical density (with examinations of carotenes) of the eluted fractions, the optical density of each fraction being read at its most pronounced absorption maximum.

For identification purposes, mixed chromatograms of pairs of carotenes were carried out as follows. Equal amounts of two carotenes were chromatographed on a relatively large amount of alumina with a low gradient of the

eluting solvent (conditions favoring a high resolution). The two carotenes were considered chromatographically identical if one smooth peak was obtained in the distribution curve and the material represented by this peak constituted a near complete recovery of the combined carotenes.

Experimental

"Skellysolve F", boiling range 40°–50° C, was used for light petroleum throughout this investigation. Melting points were measured with a Leitz heating stage microscope and were corrected. Absorption spectra in the ultraviolet and visible regions were recorded with a Spectracord (Fisher Scientific Co.). Infrared absorption spectra were recorded with a Perkin–Elmer Model 21 spectrometer equipped with a sodium chloride prism. Samples were mounted by the potassium bromide disk technique. X-Ray diffraction patterns were recorded with a Picker unit and a Philips circular camera.

Gross Examination of Unsaponifiable Matter

Samples of unsaponifiable matter (0.1–2 g) were chromatographed on alumina (activity II, 5–20 g) with stepwise elution. Melting points and infrared absorption spectra of eluted fractions indicated the presence of paraffinic hydrocarbons (eluted with light petroleum), aliphatic alcohols (50% ether in light petroleum), sterols (70% ether in light petroleum), and two unidentified substances (70% ether in light petroleum, and ether, respectively). All fractions except those eluted with light petroleum contained small amounts of carotenes.

Preparation of Reference Samples of β- and γ-Carotenes, Lycopene, and Phytoene

The unsaponifiable matter of oil obtained by extraction of carrots was chromatographed on alumina. Three fractions were collected which contained phytoene, β-carotene, and γ-carotene as their respective single carotenoid components. Lycopene was similarly obtained as the single carotenoid component in a chromatographic fraction of unsaponifiable matter of tomato oil. These fractions are referred to as the authentic samples. Their absorption maxima are given in Table I.

TABLE I

ABSORPTION SPECTRUM MAXIMA (mμ) OF CAROTENES*

Source	Phytoene	β-Carotene	γ-Carotene	Lycopene
Rust spores†	275 285 (295)‡	(428) 450 479	435 460 492	441 469 500
Authentic samples§	(275) 285 (295)	(428) 451 479	434 459 491	441 469 500
Mixed chromatograms‖	(275) 285 (295)	(428) 451 480	435 461 492	442 470 501
Reference 4	275 285 296¶	(425) 451 482¶	431 462 495	446 474 506

*Measured in light petroleum, or light petroleum containing less than 20% of ether.
†Fractions A, C, E, and F, respectively.
‡Figures in parentheses refer to inflexions.
§Obtained from carrots (phytoene, β- and γ-carotene) and tomatoes (lycopene).
‖Refers to materials recovered from the mixed chromatograms of authentic samples and fractions A, C, E, and F, respectively.
¶Actually in hexane.

Ergost-7-enol and Ergost-7-enyl Acetate from Ergosterol

Ergosterol (laboratory reagent), crystallized to m.p. 155° C, $[\alpha]_D$ −117° (*c*, 1.3, $CHCl_3$), was converted to the acetate with acetic anhydride and pyridine. The crude acetate was crystallized from a mixture of acetone and ethyl acetate to m.p. 173°–178° C (plates). This product was hydrogenated in ethyl acetate with Raney nickel at room temperature and atmospheric pressure according to Bladon *et al.* (2). Ergost-7-enyl acetate crystallized from ethyl acetate in plates, m.p. 156.5° –161° C. The acetate (ca. 90 mg) was chromatographed on alumina (activity II, 20 g) and eluted successively with gradient 0–50% ether in light petroleum (400 ml), gradient 50–100% ether in light petroleum (175 ml), and gradient 0–10% methanol in ether (240 ml). Fractions eluted with 15 to 35% ether contained ergost-7-enyl acetate (45 mg), crystallized once from acetone to m.p. 161.5°–163° C (plates), $[\alpha]_D$ −5° (*c*, 1.1, $CHCl_3$). Reported optical rotations for ergost-7-enyl acetate are −15.9° (11, 13), −5.3° (14), −4° (1), and −3° (2). Fractions eluted with 2 to 5% methanol contained ergost-7-enol (43 mg), crystallized once from acetone to m.p. 150–151° C (plates), $[\alpha]_D$ −1.6° (*c*, 0.8, in $CHCl_3$) (recorded for material recovered from the filtrate after crystallization). Infrared absorption spectra and X-ray diffraction patterns were recorded of the sterol and its acetate.

Identification of Phytoene

The oil extracted from a sample of spores which had been stored for a year at +5° C was refluxed for 1 hour with ethanolic potassium hydroxide. The unsaponifiable matter (20 mg) was isolated and chromatographed on alumina (activity II, 20 g) with gradient 0–100% ether in light petroleum (1100 ml). Phytoene was identified in the eluate of 7 to 9% ether (fraction A) by the shape and position of its absorption maxima and by a mixed chromatogram of fraction A and authentic phytoene (absorption maxima, see Table I).

A sample of spores which had been stored for 8 months at +5° C and a freshly harvested sample were similarly examined, except that the oils were saponified at a lower temperature (25° C). Phytoene was not detected; the relevant chromatographic fractions showed intense general absorption in the ultraviolet region with inflexions near 268, 273, and 278 mμ.

Identification of Five Carotenes

In the work described under this heading, carotene fractions were excluded from light and atmospheric oxygen whenever possible and kept at −30° C when not in use; solvents were evaporated below 30° C in a vacuum. A sample of freshly harvested spores (5.18 g) was extracted with ether at 0° C in the dark for 90 hours. Hydroquinone (ca. 30 mg) was added to the spores before the extraction. The oil (1.2 g) recovered from the extract was saponified at room temperature in ethanolic potassium hydroxide and the unsaponifiable matter was isolated in the usual way. The coloring matter of the unsaponifiable fraction proved epiphasic upon shaking with a mixture of equal

volumes of light petroleum and 95% methanol (aqueous). The unsaponifiable matter was chromatographed on alumina (activity II, 14 g) and eluted successively with light petroleum (100 ml), gradient 0–100% ether in light petroleum (280 ml), and gradient 0–7% methanol in ether (70 ml). Overlapping carotene fractions of the eluate were rechromatographed, using lower gradients of eluting solvents and, in one chromatogram, a less active grade (IV) of alumina. Five fractions (B, C, D, E, F) were obtained, each containing a single carotene. The carotenes contained in fractions C, E, and F were identified as β-carotene, γ-carotene, and lycopene, respectively, by the shape and position of the absorption maxima of the fractions and by their mixed chromatograms with authentic samples. Absorption maxima are given in Table I for the isolated carotene fractions, the authentic samples, the materials recovered from mixed chromatograms, and for reported (4) pure carotenes. The corresponding data relating to phytoene are included in the table.

To part of fraction B (containing ca. 0.008 mg carotene, absorption maxima (420),* 444, 471 mμ, in 25% ether), in light petroleum, was added iodine (0.0002 mg, in a 0.001% solution in light petroleum). The solution was left in diffuse daylight for 1 hour and subsequently chromatographed on alumina (activity II, 14 g) with gradient 0–60% ether in light petroleum (200 ml). Two carotene fractions were eluted which had absorption maxima 418, 442, 470 (in 30% ether), and (425), 451, 479 mμ (in 55% ether), respectively. The second fraction (eluted with 55% ether) contained β-carotene, which was identified by its absorption spectrum and a mixed chromatogram with an authentic sample. Fraction D (absorption maxima 347, 429, 454, 485 mμ, in 45% ether) was similarly isomerized with iodine and chromatographed. Three isomeric fractions were obtained, with absorption maxima 450, 480 (in 60% ether); 432, 457, 487 (in ether); and (437), 461, 491 mμ (in 1% methanol in ether), respectively. The third fraction (eluted with 1% methanol in ether) contained γ-carotene which was identified by its absorption spectrum and a mixed chromatogram with an authentic sample.

The peak quantities of the respective carotenes were contained in eluates as tabulated below (the corresponding data relating to phytoene are included):

Activity of alumina used (Brockman's scale)	Composition of eluate containing peak quantity of carotene	Fraction eluted	Identity of carotene (polyene)
II	7% ether	A	Phytoene
II	25% ether	B	*cis*-β-Carotene
II	35% ether	C	β-Carotene
II	45% ether	D	*cis*-γ-Carotene
II	55% ether	E	γ-Carotene
II	4% methanol	F	Lycopene
IV	17% ether		

*Throughout this article, absorption figures in parentheses refer to inflexions.

Isolation and Identification of Ergost-7-enol

A crude sterol fraction (0.55 g) of the unsaponifiable matter was rechromatographed on alumina (activity II, 20 g) and eluted successively with 50% ether in light petroleum (175 ml), gradient 50–100% ether in light petroleum (600 ml), and gradient 0–5% methanol in ether (125 ml). The material (0.29 g) contained in the combined eluates of 62 to 73% ether was crystallized (methanol, light petroleum, acetone) to m.p. 147°–151° C, unchanged on admixture with authentic ergost-7-enol of m.p. 150–151° C, $[\alpha]_D$ +6° (*c*, 1.9, $CHCl_3$). Reported highest melting point for ergost-7-enol, 149°–150° C (5), and optical rotations −4° (5), −2° (13), and 0° (12, 14). Calculated for $C_{28}H_{18}O$: C, 83.9%; H, 12.1%. Found: C, 84.2%; H, 12.0%. A less pure fraction (m.p. 143°–147.5° C, 76 mg) of the sterol was converted to the acetate with acetic anhydride and pyridine. The crude acetate was crystallized from ethanol to m.p. 158°–160° C (plates), unchanged on admixture with authentic ergost-7-enyl acetate of m.p. 161.5°–163° C. Reported highest melting point for ergost-7-enyl acetate, 160°–161.5° C (13). Calculated for $C_{30}H_{50}O_2$: C, 81.4%; H, 11.4%. Found: C, 81.8%; H, 11.5%. Infrared absorption spectra and X-ray diffraction patterns of the isolated sterol and its acetate were recorded.

Results and Discussions

Ether extraction of different samples of spores gave 16 to 23% of oil, in fair agreement with earlier findings (10). The composition of one sample of oil was determined as 8% unsaponifiable matter, 10% glycerol, and 82% fatty acids, including 4% of free fatty acids which were present in the oil. Chromatography of the unsaponifiable matter on alumina and examination of eluted fractions by melting point and absorption spectroscopy indicated the presence of paraffinic hydrocarbons, aliphatic alcohols, sterols, and two unidentified substances. A quantitative estimation of the five fractions was not arrived at. However, the sterol fraction may be described as being relatively large, the paraffin and alcohol fractions as intermediate, and the two unidentified fractions as small. Minor amounts of carotenoids were contained in most of the eluted fractions.

An investigation of the carotenoids confirmed the earlier findings of Irvine *et al.* (6) and further revealed the presence of a colorless polyene, phytoene, and of two *cis*-isomeric carotenes. The unsaponifiable matter of oil obtained from freshly harvested spores was fractionated by chromatography on alumina using gradient elution. This technique was preferred to stepwise elution to ensure that "false peaks" would not occur in the distribution curve of the eluted material. After rechromatography of appropriate fractions, five well-separated carotene fractions were obtained. Most of these contained, besides carotenes, other unsaponifiable material which, however, did not interfere with the subsequent identification of the carotenes. The five carotenes, in order of their elution from the column, were identified as a *cis*-isomer of β-carotene, β-carotene, a *cis*-isomer of γ-carotene, γ-carotene, and

lycopene. The identification of β-carotene, γ-carotene, and lycopene was based on their absorption spectra and their chromatographic identity with the respective authentic carotenes, as shown by mixed chromatograms. Reference samples of β- and γ-carotene and lycopene were prepared from carrots and tomatoes. The identities of the two *cis*-isomeric carotenes were established through their iodine isomerization and the subsequent isolation and identification of β- and γ-carotene respectively fom the two stereoisomeric sets produced. The configurations of most *cis*-isomeric carotenes have not been conclusively established (15). It is reasonable to assume, however, that the present isomers of β- and γ-carotene have two and one *cis*-double bonds respectively, because of the observed differences (10 and 6 mμ) between the longest wavelength maxima of the *cis*-forms and the corresponding all-*trans*-forms (15). No attempt was made to establish whether the *cis*-isomeric carotenes are metabolites of the spores or artifacts formed after extraction of the oil by isomerization of β- and γ-carotene. In this part of the work, however, light was excluded from the samples whenever possible and excessive heat was avoided in order to retard the process of isomerization.

The colorless polyene, phytoene, was found in a different sample of spores. It was eluted from an alumina column in front of the colored carotenes and similarly identified through its absorption spectrum and a mixed chromatogram with an authentic sample obtained from carrots. Phytoene was detected only in one out of three samples of spores investigated. The phytoene-containing chromatographic fraction of this one sample of spores differed from the corresponding fractions of the two other samples in that the latter fractions showed intense general absorption in the ultraviolet region. The nature of the highly absorbing material was not established. It is possible that phytoene could have been present in all three samples and that in two of them it was masked by the highly absorbing material. The related polyene, phytofluene, which is a common constituent of carotenoid-rich fungi (5), was not detected in the present investigation. Xanthophylls (hypophasic carotenoids) were absent.

The total carotenes constituted 0.12% of freshly harvested spores. The quantities of individual components are listed in Table II; values recorded by Irvine *et al.* (6) are included for comparison.

The sterol fraction of the unsaponifiable matter apparently contained only one sterol, which was identified as ergost-7-enol. The sterol was purified by further chromatography and crystallizations, and characterized by its elementary analysis, melting point, optical rotation, infrared absorption spectrum, and X-ray diffraction pattern. The acetate was prepared and similarly characterized, except that there was insufficient material for a measurement of optical rotation. For reference, ergost-7-enol and ergost-7-enyl acetate were prepared through partial hydrogenation of ergosteryl acetate according to Bladon *et al.* (2). In the course of this preparation, ergost-7-enyl acetate was found to partially hydrolyze to ergost-7-enol on passing through a chromatographic column of alumina. The recorded

properties of the rust sterol and its acetate were identical (infrared absorption spectra, X-ray diffraction patterns) or in close agreement (melting points, optical rotations) with the corresponding properties of the synthetic ergost-7-enol and ergost-7-enyl acetate. Further, the melting points of the solated sterol and its acetate were unchanged on admixture with the authentic specimens. Although a slightly high optical rotation of the rust sterol indicated that it was not entirely pure, its identity as ergost-7-enol seems well substantiated by the other evidence produced.

Ergost-7-enol has been found in various fungi, accompanied by larger amounts of ergosterol (13). In the present investigation, however, ergosterol was not detected. Synonyms of ergost-7-enol are 5:6,22:23-tetrahydroergosterol, γ-ergostenol, and fungisterol. It might be appropriate to mention that the latter name has been assigned to two different sterols occurring in fungi, namely to ergost-7-enol as found in ergot in 1908 by Tanret (11), and to ergosta-6:7,8:9,22:23-trienol which later was isolated from *Penicillium chrysogenum* by Saito (9).

TABLE II

CAROTENES (POLYENES) IN UREDOSPORES OF *Puccinia graminis tritici*, RACE 15B

Components present	Quantitative distribution*	
	As % of total carotenes present†	As p.p.m. of spores
Phytoene‡		10
cis-β-Carotene	0.6	7
β-Carotene	20.7	240 (210§)
cis-γ-Carotene	3.9	45
γ-Carotene	74	860 (490§)
Lycopene	0.8	9

NOTE: Components are listed in order of increasing adsorptive power.
*$E^{1\%}_{1cm}$. Values for phytoene (1220), β-carotene (2580), γ-carotene (2720), and lycopene (3470) were taken from reference 4. Values for *cis*-β-carotene (2000) and *cis*-γ-carotene (2100) were chosen as 77% of those for the parent all-*trans*-carotenes.
†Excluding phytoene.
‡Found in one out of three samples of spores investigated.
§Reported by Irvine *et al.* (6).

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THE TAXONOMY OF INSECT PATHOGENS RELATED TO BACILLUS CEREUS FRANKLAND AND FRANKLAND[1]

A. M. HEIMPEL AND T. A. ANGUS[2]

Abstract

The characteristics of seven crystal-forming *Bacillus* spp. are compared and an identification key is presented. It is concluded that the following changes in nomenclature are desirable: *Bacillus thuringiensis* Berliner becomes the type species *Bacillus thuringiensis* var. *thuringiensis* n. comb.; *Bacillus sotto* Ishiwata becomes *Bacillus thuringiensis* var. *sotto* n. comb.; and *Bacillus cereus* var. *alesti* Toumanoff and Vago becomes *Bacillus thuringiensis* var. *alesti*.

Two new species, *Bacillus entomocidus* n. sp. and *Bacillus finitimus* n. sp., and a new variety, *Bacillus entomocidus* var. *subtoxicus* n. sp., are described.

Introduction

Several crystal-forming bacteria, pathogenic for insects, have been isolated or reinvestigated in the past few years. All of these are similar to *Bacillus cereus* Frankland and Frankland, at least with regard to cellular and colony morphology, and are currently known under three scientific names: *Bacillus sotto* Ishiwata (3), *Bacillus thuringiensis* Berliner (5), and *Bacillus cereus* var. *alesti* Toumanoff and Vago (15). An isolate which is very similar to *B. cereus* var. *alesti* has been referred to in the literature as the "Anduze" strain (8). Smith *et al.* (11) classed certain strains which had been referred to as NRS 1328 and NRS 1124 as *B. thuringiensis* because the spore is borne in a slanted position in the sporangium.*

At the Forest Insect Laboratory, Sault Ste. Marie, Ontario, another crystal-forming bacterium has been isolated, along with other bacterial species, from larvae of the forest tent caterpillar, *Malacosoma disstria* Hbn., killed by excessive moisture while held in a plastic shipping container. This isolate (Ma-d-7000) is not pathogenic for either the tent caterpillar or the silkworm (*Bombyx mori* L.). There are several distinct differences between this bacterium and other crystal-forming bacteria.

The crystal-forming bacteria isolated have been described in various papers and several proposals have been advanced regarding their taxonomic status (1, 3, 8, 13, 14, and others). Unfortunately none of these proposals has found wide acceptance and some confusion has arisen. These organisms are of primary interest because they are pathogenic for insects, and it would be useful to insect pathologists if they could be easily and quickly differentiated.

It seemed of interest to us, therefore, to study all the known isolates of crystal-forming bacteria under identical conditions, and describe them in comparable terms. This paper reports such a study.

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*These were isolated by Steinhaus, EAS57-1-1 (NRS 1328) from *Aphomia gularis* Zell., and EAS58-1-1 (NRS 1124) from *Plodia interpunctella* Hbn. (13).

Results

The species or varieties examined have been detailed above. The media used were prepared as described by Smith *et al.* (11), and the various tests were made as recommended by them. All incubations were at 32° C unless otherwise noted.

Bacillus sotto Ishiwata

This bacterium was isolated by Ishiwata in 1902 from larvae of silkworm dying from "flacherie"; his first report was published in 1905. A subculture of the organism was sent to us in 1952 by Ono of the Sericultural Experimental Station in Tokyo, where the organism has been maintained since 1902. All existing subcultures of this bacterium are from this source.

Spore.—Oval, averaging 1.0 by 1.5 microns; subterminal; thin-walled; generally not formed before 36 hours' incubation at 28° C.

Sporangium.—Cylindrical, not swollen; spores set obliquely at one end; diamond-shaped crystalline parasporal body present in each sporangium. These parasporal bodies are toxic for certain Lepidoptera.

Vegetative rods.—Thin, relatively short rods, with round ends; average size 1.0 to 1.2 by 2.2 to 4.0 microns usually found in short chains; slightly motile; peritrichous flagellation; Gram-positive.

Gelatin.—Rapid liquefaction.

Agar colonies.—Large, rough, flat, irregular, with whiplash outgrowths. Transmitted light: creamy white; reflected light: brownish white.

Nutrient broth.—Uniform turbidity; slight soft sediment; no pellicle after 72 hours; sediment easily and evenly dispersed upon shaking.

Starch.—Hydrolyzed.

Sugar fermentation.—Acid (with ammoniacal nitrogen) from levulose, trehalose, maltose, sucrose, and glucose after 48 hours. Action variable on inulin and salicin.

Voges-Proskauer.—Acetylmethylcarbinol produced.

Phospholipase C.—Produced. No reddish pigment produced.

Bacillus thuringiensis Berliner

This organism was isolated from sick larvae of *Ephestia kühniella* Zell. in 1911 by Berliner (5). Mattes reisolated the bacterium and published a description of the disease it caused in *E. kühniella* (10). A subculture of what was believed to be Mattes' original strain was obtained from Steinhaus in 1950. Later Smith supplied us with a strain (from Porter) bearing his culture number NRS 996. Cultures from several other investigators have since been received. With one exception, however, these cultures were all distributed by Steinhaus or Smith. The exception, a culture isolated by Majumder *et al.* (9), was isolated from dying and dead larvae of *Heliothis obsoleta* (Fabr.) in Mysore, India.

These investigators were unaware (Majumder, personal communication) that De and Konar (7) had imported a culture of *B. thuringiensis* from Steinhaus for their work on the granary beetle in India. It will be seen in a later

section that *B. thuringiensis* and Majumder's isolate are remarkably similar. For this reason, the possibility that this organism escaped into the field from De and Konar's laboratory and was detected by Majumder is not to be excluded.

The description of the sporeforming bacterium isolated by Majumder *et al.* (9) is identical with the following.

Spore.—Oval, average size 1.0 by 1.5 microns; subterminal; usually formed in 24 to 48 hours; spores lie obliquely in the sporangium.

Sporangium.—Oval, not swollen; diamond-shaped to cuboidal parasporal body formed in each sporangium. These crystals are toxic for many Lepidoptera.

Vegetative rods.—Short stout rods with round ends; average size 1.7 by 4.0 microns; slightly motile to non-motile; peritrichous flagellation; Gram-positive.

Gelatin.—Slow liquefaction.

Agar colonies.—Large, dry and flat, irregular, slightly lobed border. Transmitted light: cream-white, opaque, "ground-glass" appearance; reflected light: brownish white.

Nutrient broth.—Body of broth relatively clear. Pellicle forms but breaks readily into crumbling, flaky mass that sinks quickly, and is not easily dispersed with shaking.

Starch.—Hydrolyzed.

Sugar fermentation.—Acid (with ammoniacal nitrogen) from levulose, trehalose, maltose, sucrose, and glucose after 48 hours. No acid with the above-mentioned sugars after 10 days' incubation. Action with salicin variable.

Voges-Proskauer.—Acetylmethylcarbinol produced.

Phospholipase C.—Produced. No reddish pigment produced.

Bacillus sp. (EAS57-1-1 or NRS 1328)

This bacterium was isolated from *Aphomia gularis* Zell. by Steinhaus (12). A subculture was sent to Smith, who did not name it, but classified it tentatively under *B. thuringiensis* on the basis of the slanted spores found in the sporangium (11). The organism was sent to us by Steinhaus and by Smith in 1950.

Spores.—Cylindrical with round ends, average size 1.0 by 1.6 microns; subterminal; usually formed in 48 hours; spores lie obliquely in the sporangium.

Sporangium.—Cylindrical, not swollen; thin-walled; small, diamond-shaped parasporal body formed in each sporangium. These parasporal bodies are extremely toxic for certain Lepidoptera.

Vegetative rods.—Stout rods measuring on the average 1.7 by 3.0 to 5.0 microns; ends only slightly rounded; form pairs or short chains; slightly motile to non-motile; peritrichous flagellation; Gram-positive.

Gelatin.—Slow liquefaction.

Agar colonies.—Large, flat, irregular shape, border with slight to extensive whiplash outgrowths. Transmitted light: greyish, cream-white, mottled appearance, opaque; reflected light: brownish white.

Nutrient broth.—Broth evenly turbid. Soft deposit formed after 48 hours which gives an even turbidity in the shaken tube. No pellicle formed.

Starch.—Hydrolyzed.

Sugar fermentation.—Acid (with ammoniacal nitrogen) from levulose, trehalose, sucrose, and glucose after 48 hours. No acid reaction after 12 to 14 days' incubation. Action with maltose and salicin is variable. Trehalose and glucose usually acid after 25 days' incubation.

Voges-Proskauer.—No acetylmethylcarbinol formed.

Phospholipase C.—Not produced.* No reddish pigment produced.

Bacillus sp. (EAS58-1-1 or NRS 1124)

Steinhaus isolated this bacterium from *Plodia interpunctella* (Hbn.) in 1945 (12). Smith classified it tentatively with *B. thuringiensis*, along with EAS 57-1-1. The organism was sent to us in 1950 by Smith.

Spores.—Cylindrical with round ends, average size 0.9 by 1.5 microns; subterminal; usually formed in 48 hours; spores lie obliquely in the sporangium.

Sporangium.—Cylindrical, not swollen; thin-walled; small, diamond-shaped parasporal body formed in each sporangium. These parasporal bodies are of a relatively low toxicity for some Lepidoptera.

Vegetative rods.—Relatively short, stout rod, measuring 1.6 by 3.0 to 5.0 microns; round ends; pairs or short chains; non-motile to slightly motile; peritrichous flagellation; Gram-positive.

Gelatin.—Slow liquefaction.

Agar colonies.—Large, flat, irregular-shaped; dull; border shows extensive whiplash outgrowths after 48 hours. Transmitted light: greyish, cream-white, mottled, opaque; by reflected light: brownish white.

Nutrient broth.—Broth only slightly turbid. Thin pellicle formed in 48 hours' incubation. Pellicle breaks into flakes upon shaking.

Starch.—Hydrolyzed.

Sugar fermentation.—Acid (with ammoniacal nitrogen) from levulose, trehalose, maltose, sucrose, and glucose after 48 hours. No acid reaction in these sugars after 12 to 14 days.

Voges-Proskauer.—No acetylmethylcarbinol produced.

Phospholipase C.—Not produced.* No reddish pigment produced.

Bacillus cereus var. *alesti* Toumanoff and Vago.

This bacterium was isolated from silkworm larvae dying of "flacherie", in the Cévennes region in France (15). All subcultures in existence came originally from Toumanoff.

Spores.—Oval, average size 1.0 by 1.5 microns; subterminal; usually formed in 24 to 48 hours; spores lie obliquely in the sporangium.

*First noted by E. A. Steinhaus (12).

Sporangium.—Oval, not swollen; thin-walled; diamond-shaped parasporal body formed in each sporangium. These crystals are very toxic for many Lepidoptera.

Vegetative rods.—Short, stout rods with round ends; average size 1.0 by 5.1 microns; separate or form couplets; non-motile to slightly motile; peritrichous flagellation; young cells stained with Gram's stain appear foamy; Gram-positive.

Gelatin.—Slow liquefaction.

Agar colonies.—Large, shiny and flat, slightly raised, irregular. Transmitted light: creamy white, granular; reflected light: brownish white.

Nutrient broth.—Evenly turbid. A sediment forms which disperses evenly upon shaking.

Starch.—Hydrolyzed.

Sugar fermentation.—Acid (with ammoniacal nitrogen) from levulose, trehalose, maltose, and glucose after 48 hours. Acid from sucrose in approximately six days. No acid reaction after 14 days in any sugars tested.

Voges-Proskauer.—Acetylmethylcarbinol produced.

Phospholipase C.—Produced. Red pigment produced in egg-yolk agar plates or in injected egg yolks.

Bacillus sp. (strain "Anduze" isolated by Vago in 1952)

This bacterium was isolated from leaf litter in silkworm rearings by Vago and was sent eventually to DeLaporte and Béguin, who examined the bacterium and reported that they thought it should be grouped with *B. thuringiensis* with *B. cereus* var. *alesti* and *B. sotto.* The strain called "Anduze" was sent to us by DeLaporte and Béguin in 1956 (8).

Spores.—Oval, average size 0.7 to 0.8 by 1.5 microns; slightly slanted in the sporangium; usually formed in 48 hours.

Sporangium.—Oval, not swollen, thin-walled; small diamond-shaped parasporal body formed in each sporangium. These crystals are very toxic for certain Lepidoptera.

Vegetative rods.—Stout, relatively long rods, with square to slightly rounded ends; average size 1.3 by 5.5 microns; rods found separately or in couplets; non-motile to slightly motile; peritrichous flagellation; Gram-positive.

Agar colonies.—Medium to large, rough, flat, irregular. Transmitted light: creamy white, granular, opaque; reflected light: brownish white.

Nutrient broth.—Broth evenly turbid. Considerable deposit disperses evenly when shaken.

Starch.—Hydrolyzed.

Sugar fermentation.—Acid (with ammoniacal nitrogen) from levulose, trehalose, maltose, and glucose after 48 hours. No acid from sucrose. When held for 2 months on agar slants containing sucrose the sugar was fermented; however upon return for 2 weeks to nutrient agar slants the strain reverted to a non-sucrose fermenting type. Since DeLaporte and Béguin report a positive sucrose reaction this may be considered a variable reaction by this strain.

Voges-Proskauer.—Acetylmethylcarbinol produced.

Phospholipase C.—Phospholipase produced. A reddish pigment is produced on egg-yolk agar or when injected into egg yolks. The amount of pigment is slightly less than that produced by "*alesti*".

Bacillus sp. (Heimpel's number Ma-d-7000)*

The bacterium was isolated from dead larvae of the tent caterpillar, *Malacosoma disstria* Hbn., in 1956, at Sault Ste. Marie.

Spores.—Cylindrical, with round ends; average size 1.0 by 1.5 to 2.0 microns; subterminal; usually formed in 48 hours; spores do not lie obliquely in the sporangium.

Sporangium.—Cylindrical, not swollen; thick-walled; a diamond- to irregular-shaped parasporal body is formed in the sporangium along with the spore. The sporangium does not lyse; the spore and parasporal body are always found attached to one another.

Vegetative rods.—Long, stout rods with slightly rounded ends; average 2.0 by 5.0 to 6.0 microns in size; rods separate, in couplets, or in even- and odd-numbered chains of up to eight bacteria. Non-motile to slightly motile; peritrichous flagellation; Gram-positive.

Gelatin.—Slow liquefaction.

Agar colonies.—Large, slightly raised, flat, shiny, irregular, border shows fine, whip-like dentation. Transmitted light: cream-white; reflected light: brownish white.

Nutrient broth.—Slight growth in broth; heavy pellicle formed in 48 hours; pellicle breaks into flakes upon shaking.

Starch.—Not hydrolyzed.

Sugar fermentation.—Acid (with ammoniacal nitrogen) from levulose, trehalose, maltose, glucose, sucrose, and salicin after 48 hours. Variable reaction from dextrin. No acid reaction from any of the sugars tested after 14 days' incubation.

Voges-Proskauer.—Acetylmethylcarbinol produced.

Phospholipase C.—Produced. No reddish pigment produced.

There are several characteristics common to all of these isolates: they convert nitrates to nitrites and utilize citrate slowly as a carbon source; all grow well in the range 28–35° C; all grow anaerobically in the presence of glucose; none of the bacteria ferment xylose, arabinose, mannitol, mannose, lactose, melezitose, dulcitol, raffinose, sorbitol, galactose, rhamnose, inositol, melibiose, or dextrin, even after 30 days' incubation.

There is a clear-cut distinction between these bacteria in relative toxicity for silkworm larvae which from the pathologists' point of view is most important. If the bacteria are grown under identical conditions until sporulation (when crystals are produced), are harvested and washed in water, and are dried and fed in weighed amounts to silkworm larvae, significant differences

*Isolated by Mrs. D. MacNamee at the Forest Insect Laboratory.

in toxicity are found. The results of a typical experiment are given in Table I. The characteristic symptom is a profound irreversible paralysis that ends in death. It has been shown elsewhere (1) that the time elapsing between ingestion and the appearance of symptoms is inversely proportional to the size of the ingested dose and of course the titer. However, no matter how heavy the dose, paralysis does not occur in under an hour.

From the table it can be seen that the minimal paralytic dose (MPD) for *Bacillus* sp. (EAS 57-1-1), *B. sotto*, and *B. cereus* var. *alesti* is less than 1 μg; the limit of accuracy of the testing method is 0.5 μg. The MPD for both *B. thuringiensis* strains (Mattes' and Majumder's) is not less than 150 μg. The organism *Bacillus* sp. (EAS 58-1-1) is even less toxic, and *Bacillus* sp. (Ma-d-7000) is not toxic for the silkworm. We see, therefore, in these organisms a complete range of toxicity for the silkworm. The values are not absolute but vary slightly with different subculturing of bacteria. The stage at which the insect is tested may also affect the MPD. However, the order of virulence is invariable, EAS 57-1-1, *sotto*, and *alesti* being always many times more toxic than the other strains tested.

TABLE I

THE TOXICITY OF VARIOUS *Bacillus* SPP. FOR SILKWORM LARVAE

Bacterium	Dose in μg	Larvae tested	Hours till paralysis	Hours till death
Bacillus sp. (EAS 57-1-1)	1	20	3	†
Bacillus sotto	1	20	3	†
Bacillus cereus var. *alesti*	1	20	3	†
Bacillus thuringiensis	150	20	No paralysis	18
(Mattes' str.)	Massive*	20	6	†
Bacillus thuringiensis	150	20	No paralysis	18
(Majumder's str.)	Massive	20	6	†
Bacillus sp. (EAS 58-1-1)	150	20	No paralysis	24
	Massive	20	9	†
Bacillus sp. (Ma-d-7000)	150	20	No paralysis	No death
	Massive	20	No paralysis	No death

*Massive dose, considerably more than 150 μg of culture.
†General paralysis invariably leads to death; time of death cannot be accurately determined.

Discussion

The results of these studies and those of others (DeLaporte and Béguin (8), Toumanoff (14), Steinhaus (13), Toumanoff and Vago (15)) have persuaded us that the three bacterial isolates previously referred to by the specific epithets *sotto*, *alesti*, and *thuringiensis* are so similar that they must be regarded as one species.

However there are consistent differences, mostly minor in nature, that justify designating them as varieties. The question arises which is the earliest, legitimate combination of names. Although the "sotto-Bacillen" was isolated, described, and so named by Ishiwata in 1902, it was not until

July, 1915, that the correct combination "*B. sotto*" was used (2, 4); in this paper, Aoki and Chigasaki cited Ishiwata's paper containing the description of the bacterium. Aoki and Chigasaki have thus satisfied the requirements set out in principle 12 of the Bacteriological Code of Nomenclature, regarding the valid publication of the name of any taxonomic group.

There is a rule in the Bacteriological Code that applies directly to such a situation:

Excerpt from rule 12: "The date of a name or of an epithet is that of its valid publication. For purposes of priority, however, only legitimate names and epithets published in legitimate combinations are taken into consideration. In the absence of proof to the contrary, the date given in the work* containing the name or epithet must be regarded as correct."

Berliner's paper (5) was published in April, 1915; therefore there can be no doubt that the combination *Bacillus thuringiensis* Berliner precedes that of *Bacillus sotto* Ishiwata, although the priority is established by a narrow margin of three to four months.

We therefore propose that *Bacillus sotto* be renamed *Bacillus thuringiensis* var. *sotto* n. comb. and that *Bacillus cereus* var. *alesti* be named *Bacillus thuringiensis* var. *alesti* n. comb. Should any worker disagree with the use of *Bacillus thuringiensis* the alternative is to petition the Bacteriological nomenclature committee and request a decision. We agree with DeLaporte and Béguin (8) that *alesti* and the "Anduze" strains are very similar and therefore we include the "Anduze" strain with the new combination *B. thuringiensis* var. *alesti*. To comply with rule 7 of the Bacteriological Code *Bacillus thuringiensis* Berliner must be renamed *Bacillus thuringiensis* var. *thuringiensis* n. comb.†

Since the *Bacillus* spp., strains EAS 57-1-1 and EAS 58-1-1, produce neither phospholipase C nor acetylmethylcarbinol, it is clear that they cannot be classed either with *B. thuringiensis* or *B. cereus*. Since they do not ferment xylose or arabinose and do not resemble *B. megaterium* in any other way, the alternative is to identify this organism as a new type. Thus we propose that the name *Bacillus entomocidus* var. *entomocidus* n. sp. be applied to the bacterium bearing the culture number EAS 57-1-1. The culture bearing the number EAS 58-1-1 then should be named *Bacillus entomocidus* var. *subtoxicus* n. comb. The criterion for establishing species and variety status in the latter case is relative toxicity for silkworm larvae. In many pathogens toxicity is variable; in our experience this is not the case with crystal-forming bacteria pathogenic for Lepidoptera.

One other type known by Heimpel's culture number Ma-d-7000 is sufficiently different from the other crystal-formers to necessitate naming it as a separate species. Since the parasporal bodies invariably remain attached to

*Rule 11 of the Code states that for purposes of priority, the legal date is the effective date of publication of the journal, not the date the manuscript was accepted for publication.

†Rule 7 of the International Bacteriological Code of Nomenclature reads as follows: "If the species is divided into subspecies, the subspecific epithet of the subspecies containing the type of species shall be the same as that of the species."

the spores and since the inclusions are not known to be toxic for Lepidoptera, it is proposed that the culture Ma-d-7000 be named *Bacillus finitimus* n. sp.

Using the criteria discussed above it is possible to create a key that permits easy differentiation of these species and others that resemble *B. cereus*. Such a key, in the style of those in *Bergey's Manual*, is presented below (6).

KEY TO THE SPECIES OF THE "*Bacillus cereus* GROUP"

Mesophilic (good growth between 28° and 35° C), aerobic (usually facultative anaerobes). Spores ellipsoidal to cylindrical, paracentral to subterminal, walls thin. Sporangia not distinctly bulged. Gram-positive.

A. No parasporal body present

(*a*) Acid from xylose and arabinose with ammoniacal nitrogen. Acetylmethylcarbinol not produced. Phospholipase C not produced.

1. *Bacillus megaterium*

(*aa*) No acid from xylose and arabinose. Acetylmethylcarbinol produced. Phospholipase C produced.

(*b*) Saprophytic, sometimes pathogenic but not causing anthrax; sometimes motile.

(*c*) Growth on agar not rhizoid.

2. *Bacillus cereus*

(*cc*) Growth on agar rhizoid; seldom motile.

3. *Bacillus cereus* var. *mycoides*

(*bb*) Pathogenic. Causative agent of anthrax; non-motile.

4. *Bacillus anthracis*

B. Parasporal bodies present

(*a*) Parasporal body released from sporangium and separated from the spore in two to six days; pathogenic for Lepidoptera larvae.

(*b*) Acetylmethylcarbinol produced. Phospholipase C produced.

(*c*) Pellicle formed in nutrient broth. Little turbidity in body of broth. Pellicle breaks into flakes in shaken culture. Low toxicity for the silkworm.

5. *Bacillus thuringiensis* var. *thuringiensis*

(*cc*) No pellicle formed in nutrient broth, body of broth evenly turbid, shaken culture gives even dispersal. Highly toxic for Lepidoptera.

(*d*) No pigment formed when grown on egg-yolk agar.

6. *Bacillus thuringiensis* var. *sotto*

(*dd*) Rosy pigment formed in agar after several days' growth on egg-yolk agar.

7. *Bacillus thuringiensis* var. *alesti*

(*bb*) No acetylmethylcarbinol produced. No phospholipase C produced.

(*c*) Acid from trehalose, levulose, and glucose after 20 days' incubation at 32° C. Highly toxic for many Lepidoptera.

8. *Bacillus entomocidus* var. *entomocidus**

(*cc*) No acid from trehalose, levulose, and glucose after 20 days. Low toxicity for certain Lepidoptera.

9. *Bacillus entomocidus* var. *subtoxicus*

(*aa*) Parasporal body firmly attached to spore, even after months of storage. Acid from cellobiose after 48 hours' incubation. Non-pathogenic for certain Lepidoptera.

10. *Bacillus finitimus*†

There are some differences between our results and the results reported by others in the literature. However, since in some cases we used different media and reagents, some variation is to be expected. A careful comparison has shown that discrepancies are of a minor nature and do not affect the main purpose of this paper.

*Entomon, insect; caedo, to kill.

†Finitimus, neighbor.

This survey of "*B. cereus* group" organisms that produce parasporal bodies reveals a heretofore unexpected variance, both in relation to the toxicity of the various species for insects, and in their biochemical reactions. However, since the means of identifying bacteria are arbitrary and entirely artificial, it is not surprising that a group of apparently similar organisms should have such distinct dissimilarities. Using the present taxonomic system it is necessary to treat the bacteria described herein as we have recommended.

One other significant observation should be noted to aid those interested persons who lack the facilities for comparative studies. We have found that, when grown in nutrient broth cultures, all crystal-bearing species (*sotto*, *alesti*, *entomocidus*) that grow evenly through the body of the broth and produce little or no pellicle in the first 36 hours are extremely toxic for silkworm larvae; usually the MPD is of the order of 1 μg per gram of insect. The species that produce heavy pellicles (that, on shaking, disperse as persistent flakes) under the same conditions are very much less toxic for silkworm larvae.

It has been reported by Toumanoff (16) and Vaňková (17) that some crystal-forming bacteria will, under certain special conditions, cease producing crystals. Fitz-James and Young (personal communication) mentioned that ". . . by the means of a mutagenic agent it is possible to obtain acrystallophoric variants from crystal-forming strains . . .". Toumanoff further stated that after passagings in wax-moth larvae there was a reversion to crystal-formation. It is difficult to decide what is the taxonomic status of strains derived after unusual treatment and our identification key was not designed to solve such problems. The classification proposed and the key are based on growth under standard conditions.

Shortly after the studies described above were completed, Dr. J. Vaňková kindly supplied a culture of the crystal-forming sporeformer isolated, by Dr. Weiser, from the sweet pepper moth *Plodia interpunctella* (17). We have made preliminary examination of this isolate as described above and agree with Vaňková that it is a strain of *Bacillus thuringiensis* var. *thuringiensis*.

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EMPLOI D'UN MILIEU SYNTHÉTIQUE SIMPLIFIÉ POUR LA CULTURE IN VITRO DE CELLULES ANIMALES ET DU VIRUS DE LA POLIOMYÉLITE[1]

ROBERT DUBREUIL ET VYTAUTAS PAVILANIS

Abstract

The nutritive medium SN-3 is composed of Hanks's balanced salt solution with ribose, glutamine, and cysteine added; the glucose concentration is doubled. This nutritive solution, without additional ingredients, can keep alive monkey or mouse kidney tissue cultures during a period of several weeks. When this medium is supplemented with serum, it stimulates the in vitro cellular proliferation of freshly trypsinized monkey kidney tissue and supports the growth of HeLa cells.

We have used this simplified synthetic medium for the preparation of experimental lots of poliomyelitis vaccine. The virus concentration of the suspension of the three virus strains, Mahoney, MEF-1, and Saukett, produced with this new medium has been found as high as the concentration obtained with the more complex nutritive media usually used. We did not observe any important differences between the vaccine prepared with the SN-3 medium and that prepared with the usual media.

Introduction

Depuis quelques années, un certain nombre de milieux nutritifs de composition chimique définie (2, 6, 11, 12, 13) ont été proposés pour la culture des cellules animales in vitro. À toutes ces différentes formules, des suppléments nutritifs complexes tels que du sérum ou des extraits embryonnaires doivent cependant être ajoutés afin d'obtenir une prolifération cellulaire soutenue ou même une survivance assez prolongée de ces cultures.

Dans la préparation du vaccin contre la poliomyélite nous utilisons depuis quelques années la solution M-150 (10), modification de la solution 199 de Morgan, Morton et Parker (11), comme milieu nutritif des cellules rénales de singe servant à la multiplication du virus poliomyélitique. Pour la production du vaccin, ce milieu de composition chimique définie est utilisé sans addition de sérum ou de tout autre extrait d'origine biologique. Ce milieu suffit pendant une période d'environ 15 jours à maintenir les fragments de tissu rénal dans un état de survie suffisante pour permettre la multiplication du virus poliomyélitique.

Nous avons tenté de substituer au milieu M-150 un autre milieu de culture de composition beaucoup plus simple et de préparation plus facile et plus économique. Ce milieu devait rester de composition chimique bien déterminée afin de ne pas introduire dans le vaccin des produits susceptibles de sensibiliser les sujets vaccinés.

[1]Manuscrit reçu le 24 juin 1958.
Contribution de l'Institut de Microbiologie et d'Hygiène de l'Université de Montréal, Montréal, Qué. Ce travail a été effectué avec l'aide d'une subvention fédérale-provinciale à la recherche sur la santé publique, octroyée par le Ministère de la Santé de la Province de Québec.

Après plusieurs essais, nous nous sommes arrêtés à une solution désignée SN-3 (solution nutritive No 3) qui, employée dans la préparation du vaccin contre la poliomyélite, permet d'obtenir des suspensions virulentes de titre comparable à celui des suspensions produites avec le milieu M-150. Le vaccin obtenu par l'inactivation de ces suspensions virulentes par le formol est semblable au vaccin régulier à base de milieu M-150 en ce qui concerne le pouvoir immunologique et l'absence de toute infectivité poliomyélitique.

Ce milieu de culture SN-3 a aussi été étudié quant à son pouvoir de maintenir différentes souches de cellules animales en état de survie et aussi, avec l'apport d'un produit naturel comme le sérum, quant à son pouvoir de promouvoir la prolifération cellulaire.

Composition du milieu SN-3

La composition du milieu SN-3 est présentée au tableau I. C'est essentiellement la solution saline physiologique de Hanks (7) complétée par quelques produits organiques: le ribose et les composés aminés cystéine et glutamine; la concentration en glucose est doublée. Le pH du milieu est maintenu près de 7.2 par la présence de bicarbonate de soude.

TABLEAU I

COMPOSITION DU MILIEU DE CULTURE SN-3

Sels minéraux	g/l.	Glucides	g/l.
NaCl	8.0	Glucose	2.0
KCl	0.4	*d*-Ribose	0.03
KH_2PO_4	0.06	Produits aminés	
Na_2HPO_4	0.06	*l*-Cystéine	0.2
$MgSO_4.7H_2O$	0.1	*l*-Glutamine	0.2
$MgCl_2.6H_2O$	0.1	Indicateur de pH	
$CaCl_2$	0.14	Rouge phénol	0.02
$NaHCO_3$	1.4	Antibiotiques	
		Pénicilline	200,000 U.I.
		Dihydrostreptomycine	0.2

La plupart des produits organiques qui entrent dans la composition des milieux de cultures synthétiques déjà proposés (2, 6, 11, 13) ne sont pas utilisés dans la préparation du milieu SN-3. Les vitamines, coenzymes, bases puriques et pyrimidiques sont entièrement supprimés; les acides aminés sont réduits à la seule présence de la cystéine.

Le milieu 199 de Morgan, Morton et Parker (11) comprend plus de soixante composés chimiques; ces auteurs soulignent cependant que nombre de composants de leur milieu sont utilisés sans que leurs effets positifs sur la survie ou la croissance des cellules in vitro soient clairement démontrés. C'est le cas en particulier des suppléments vitaminiques, de l'ATP, de l'acide adénylique. Healy, Fisher et Parker (8), lors de la formulation du milieu 703, dérivé du milieu 199, trouvent que les bases puriques et pyrimidiques sont apparemment inutiles (cytosine) ou même toxiques (adénine, guanine, thymine et

uracil); ces mêmes auteurs soulignent l'effet particulièrement favorable de la cystéine. Déjà en 1948, Fischer (5) avait mis en évidence le besoin primordial des cultures tissulaires en cystine. Dans un autre travail, cet auteur (6) avait reconnu que la présence de glutamine, en l'absence d'acide glutamique, favorise grandement la survie des tissus en culture. De même, Morgan, Morton et Parker (11) ont constaté que la présence de cet amide dans le milieu 199 favorise davantage la croissance cellulaire, même en présence de tous les acides aminés connus.

Des milieux synthétiques déjà proposés pour la culture des cellules animales, le milieu SM-1 de Rappaport (12) est celui qui par sa simplicité se compare davantage au milieu SN-3. Ce milieu de Rappaport consiste en une solution saline physiologique additionnée des suppléments organiques suivants: *l*-cystéine, *l*-isoleucine et *d*-ribose; de plus, des sels de zinc, de fer, de cobalt, de manganèse et de cuivre sont ajoutés en très faible quantité. La stabilité du pH est assurée par la présence de bicarbonate de sodium et de tris(hydroxyméthyl)aminométhane.

Les essais effectués avec le milieu de culture SN-3 ont porté sur le maintien ou la croissance de cultures de cellules en feuillets monocellulaires ou de cultures de suspension de fragments tissulaires. Les résultats ainsi obtenus ont été appréciés en regard des résultats obtenus avec des préparations analogues cultivées dans le milieu M-150 et le milieu à l'hydrolysat de lactalbumine de Melnick (9).

Croissance cellulaire dans le milieu SN-3

Comme tous les milieux synthétiques déjà proposés, le milieu SN-3 doit être enrichi de sérum ou d'autres produits biologiques équivalents pour promouvoir la multiplication cellulaire. Cette capacité du milieu SN-3 complété de sérum de cheval ou de veau, à soutenir la prolifération cellulaire, a surtout été étudiée dans les cultures de cellules rénales de singe, *Maccaca mulatta*.

La préparation de ces cultures consiste à découper le cortex rénal en minces fragments de 0.5 à 1 mm d'épaisseur, et à incuber ces fragments à 6–8° C pendant environ 18 heures dans une solution de trypsine à 0.25%, selon la technique proposée par Bodian (1). Après centrifugation, les cellules rénales dissociées sont resuspendues dans le milieu de culture étudié à raison de 150,000 cellules par millilitre. Des volumes de 100 ml de cette suspension sont inoculés dans des bouteilles de Roux.

Après une période d'incubation de 5 jours à 37° C les cellules inoculées se sont multipliées au point de former un feuillet monocellulaire recouvrant toute la paroi inférieure de la bouteille de culture. Ce résultat s'obtient aussi bien avec le milieu SN-3 qu'avec le milieu M-150 ou le milieu à base d'hydrolysat de lactalbumine, pourvu que ces différentes préparations contiennent un apport suffisant en sérum, soit 2%. Toutes ces cultures peuvent survivre pendant plusieurs semaines si l'on prend soin de renouveler le milieu nutritif lorsque son pH atteint 7.0.

Le milieu SN-3 contenant 2% de sérum de veau a aussi été utilisé pour des préparations analogues de cultures de cellules rénales de souris. Les proliférations cellulaires observées ont été très satisfaisantes. De même, au cours de quelques essais effectués avec des sous-cultures de cellules HeLa, la croissance cellulaire apparaît normale lorsque le milieu SN-3 est complété avec du sérum de veau dans la proportion de 10%.

Survie des cultures de cellules ou de tissus

L'intérêt de l'emploi d'un milieu de culture synthétique, c'est-à-dire de composition chimique bien définie, dans la culture des cellules animales, réside d'une part dans la préparation de vaccins contenant un minimum de substances sensibilisantes, et d'autre part dans les possibilités d'étudier avec plus de précision le métabolisme de ces cellules.

Nous avons utilisé le milieu SN-3, à l'exclusion de tout autre supplément nutritif avec les suspensions de fragments de tissu rénal de singe cultivées selon la technique de Farrell *et al.* (4), et dans les cultures de cellules trypsinisées de reins de singe en feuillets monocellulaires. Ces cultures, préparées en vue de la production ou du contrôle du vaccin poliomyélitique, nous ont permis de constater que ce milieu suffit à la survie des fragments rénaux maintenus en suspension pendant au moins 15 jours, et à la survie des cultures des feuillets cellulaires au moins 30 jours. Au cours de ces opérations, le milieu était renouvelé après chaque période d'incubation de 7 jours.

Multiplication virale dans les cultures de tissus

Eagle et Habel (3) dans un travail sur la multiplication du virus poliomyélitique dans des cultures de cellules HeLa ont démontré que le glucose, et plus encore la glutamine et les sels minéraux sont les seuls composés requis pour la multiplication de ce virus. Les résultats que nous avons obtenus avec les cultures de cellules rénales de singe dans le milieu SN-3 concordent avec les conclusions de ces auteurs.

Des souches représentatives des trois types de virus poliomyélitique, soit les souches Mahoney, MEF-1 et Saukett, ont été cultivées sur des suspensions de fragments rénaux (4) et sur des feuillets monocellulaires provenant de la croissance de cellules rénales dissociées par l'action de la trypsine.

Les suspensions de fragments rénaux ont été préparées dans des bouteilles de Povitski à raison d'environ cinq grammes de tissue dans 500 ml de milieu de culture. Ces suspensions ont été maintenues en agitation régulière pendant 5 jours. Après cette période, le milieu de culture a été rejeté et remplacé par 800 ml de milieu frais. Deux millilitres de suspension virulente de titre $10^{6.8}$ à $10^{7.2}$ DCP 50/ml ont alors été inoculés dans chacune des cultures, et celles-ci ont été incubées à 37° C avec agitation pendant une nouvelle période de 5 jours. Le tableau II présente les titres des suspensions virulentes ainsi obtenues dans les cultures avec le milieu SN-3 en regard des titres déterminés pour des cultures parallèles effectuées avec le milieu M-150.

Ces titres infectieux sont déterminés par inoculation à des cultures en tubes de cellules rénales de singe et sont exprimés en doses cytopathogènes 50/ml. Il n'apparaît pas de différence significative entre le rendement en virus des cultures tissulaires préparées avec l'un ou l'autre des milieux étudiés.

Les cellules rénales de singe cultivées en feuillet monocellulaire directement sur le verre présentent une sensibilité égale au virus de la poliomyélite, que le milieu de culture utilisé soit le SN-3, le M-150 ou le milieu à base d'hydrolysat de lactalbumine. En effet, on obtient les mêmes résultats avec l'un ou l'autre de ces milieux nutritifs lorsque l'on détermine le titre infectieux de suspensions virulentes au moyen de dilutions sériées inoculées dans des tubes de culture de cellules trypsinisées de rein de singe. Quel que soit le type du virus, Mahoney, MEF-1 ou Saukett, les milieux de cultures étudiés n'affectent pas la sensibilité des cultures cellulaires envers l'action cytopathique de ce virus.

TABLEAU II

MULTIPLICATION DU VIRUS DE LA POLIOMYÉLITE

	Type I		Type II		Type III	
	Titre infectieux	Nombre de cultures	Titre infectieux	Nombre de cultures	Titre infectieux	Nombre de cultures
Milieu SN-3	$10^{6.75}$	8	$10^{6.62}$	9	$10^{6.81}$	7
Milieu M-150	$10^{6.74}$	16	$10^{6.56}$	14	$10^{6.97}$	14

Production du vaccin poliomyélitique avec le milieu SN-3

Nous avons tenté de préparer quelques lots de vaccin contre chacun des trois types de virus poliomyélitique en utilisant le milieu de culture SN-3. Ces lots expérimentaux d'environ 10 litres, produits avec les souches Mahoney, MEF-1 ou Saukett, ont été préparés selon une technique se rapprochant le plus possible de la technique utilisée dans la production des lots de vaccin monovalents réguliers d'environ 120 litres. Cette préparation est conforme aux recommandations du gouvernement des États-Unis (14) concernant la production du vaccin contre la poliomyélite.

Le tableau III résume les principales données obtenues avec les lots de vaccin produits avec le milieu SN-3 en regard des données correspondantes concernant les lots réguliers de vaccin produits pendant la même période de temps avec le milieu M-150.

Le taux d'inactivation du virus par la formaline à la dilution 1:4000, à 37° C est de 10 à 20% plus élevé avec les suspensions virulentes préparées dans le SN-3.

Nous avions adopté pour la production des lots réguliers de vaccin une période d'incubation de 12 jours (environ 288 heures), soit environ quatre fois la durée du temps requis pour que le pouvoir infectieux de la suspension

TABLEAU III

PRODUCTION DE VACCIN POLIOMYÉLITIQUE AVEC LES MILIEUX M-150 ET SN-3

			Temps d'inactivation requis pour atteindre un titre de 10° DCP No 50/ml		Temps total d'inactivation				
		Lot No	Heures	Moyenne	Heures	Moyenne	Innocuité*	Antigénicité†	Moyenne géométrique
Type I (Mahoney)	Milieu M-150	73	67		282		—	1/90	
		74	71	68	287	283.8	—	1/42	1/56
		75	66		282.5		—	1/46	
	Milieu SN-3	L4	60	59.3	336	332.2	—	1/145	1/111
		L6	56		329		—	1/85	
Type II (MEF-1)	Milieu M-150	62	63		329		—	1/77	
		64	68		286		—	1/144	
		65	64		288		—	1/62	
		66	66	68.6	287.5	292.8	—	1/86	1/99
		67	68		286.5		—	1/55	
		68	74		288		—	1/132	
		69	77		284.5		—	1/111	
	Milieu SN-3	L5	63.5		330.5		—	1/16	
		L8	59.5		332		—	1/34	
		L10	66.5	63.5	336	332.8	—	1/95	1/32
		L11	64		334		—	1/19	
		L12	64		331.5		—	1/39	
Type III (Saukett)	Milieu M-150	61	66.5		286.5		—	1/89	
		63	63		287.5		—	1/172	
		70	78		288		—	1/256	1/97
		71	67	69.6	281	285.8	—	1/104	
		76	72		285.5		—	1/60	
		77	71		286.5		—	1/34	
	Milieu SN-3	L1	55.5		334		—	1/78	
		L3	40.5	52.3	333.5	332.3	—	1/96	1/50
		L9	61		329.5		—	1/17	

*Essais in vitro sur des cultures cellulaires et in vivo sur le singe.
†Dilution des sérums de singe immunisé neutralisant 40 DCP 50/ml de virus homotypique.

virulente diminue jusqu'à 10° DCP 50/ml. Cette période d'inactivation permet de conserver un titre immunologique satisfaisant tout en assurant une absence complète d'infectivité; les essais d'innocuité effectués sur des cultures de tissus aussi bien que sur les singes se sont avérés toujours négatifs avec les échantillons prélevés après 9 et 12 jours d'incubation pour plus de quarante lots consécutifs.

La période d'inactivation adoptée pour les lots expérimentaux de vaccin produit avec le milieu SN-3 a été de 14 jours, soit environ 336 heures. D'après les données que nous possédons maintenant, cette période d'incubation a été inutilement prolongée; il semble même qu'au contraire, un temps d'inactivation de moins de 12 jours pouvait assurer une sécurité suffisante puisque le taux d'inactivation se trouve accéléré dans ces conditions. Malgré cette prolongation dans le temps d'inactivation, le pouvoir immunologique de ces lots expérimentaux reste en général satisfaisant. La moyenne géométrique des titres antigéniques des lots expérimentaux est plus élevée pour le type I, et plus faible pour les types II et III. Cependant, si l'on considère la variabilité très importante de ces déterminations du titre antigénique effectuées sur 12 singes selon la technique officielle proposée (14), on peut difficilement tirer des conclusions précises de ces déterminations portant sur quelques lots de vaccin de chaque type seulement. Avec les données recueillies jusqu'à ce jour, nous croyons que le pouvoir antigénique du vaccin produit soit avec le milieu SN-3, soit avec le milieu M-150, et inactivé pendant une même période de temps, devrait être du même ordre. Des travaux sont actuellement en cours pour confirmer ce point.

Conclusions

Il est possible de simplifier considérablement le milieu nutritif employé pour la culture du tissu rénal de singe et la multiplication du virus poliomyélitique dans ces cultures, en vue de préparer un vaccin contre la poliomyélite. Ainsi le milieu SN-3 tout en conservant les avantages d'un milieu synthétique, i.e. de composition chimique définie, permet d'obtenir des suspensions virales dont la concentration en antigène est comparable à celle qui s'obtient avec des milieux de culture beaucoup plus complexes. Ce milieu nutritif consiste essentiellement en une solution saline physiologique additionnée de glucose, de ribose, de glutamine et de cystéine. C'est un milieu peu coûteux et de préparation relativement facile.

Ce milieu de culture peut aussi être utilisé pour la croissance des cultures cellulaires à condition d'être enrichi de sérum comme tous les autres milieux de culture synthétiques actuellement formulés. Lorsque l'apport en sérum ou en tout autre produit naturel se trouve exclu, ce milieu reste capable de maintenir en survie, pendant une période relativement prolongée, des cultures de cellules animales. En plus de ses avantages pratiques de simplicité et de facilité de préparation, il offre ainsi des possibilités de recherche plus précises sur les divers aspects du métabolisme de ces cultures de tissu in vitro.

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ON THE FORMATION OF PROTOPLAST-LIKE STRUCTURES FROM STREPTOMYCETES[1]

R. J. Douglas, J. B. Robinson, and C. T. Corke

Abstract

Osmotically sensitive protoplast-like structures were produced by the action of lysozyme on streptomycete mycelium suspended in a buffered sucrose medium. A comparison was made of the surface of the mycelium and that of the "protoplasts" by virus adsorption and serological techniques. Although the "protoplasts" did not possess the phage receptor sites present on the original hyphae they did contain antigens in common with the cell walls. These data indicated that the structures did not have properties exactly comparable to those which have been described for protoplasts from *Bacillus megaterium*.

Introduction

In recent years a considerable body of data has been obtained on the action of lysozyme on microorganisms. A recent review by Salton (9) summarizes much of the current information concerning the nature of lysozyme, mode of action, substrate attacked, and the potential usefulness of the enzyme in the study of microbial cell structure.

The observations of Romano and co-workers (7, 8) indicated that the cell walls of species of the genus *Streptomyces* were sensitive to lysozyme action. These reports coupled with the description of protoplast formation from *Bacillus megaterium* by Weibull (12) suggested that similar structures might be produced from *Streptomyces*. The present paper describes such a transformation and attempts to assess the implications of these observations.

Materials and Methods

The *Streptomyces scabies* cultures used were stocks of proved pathogenicity originally obtained from potato tuber lesions at the Laboratory of Plant Pathology, St. Catherines. The other species used were obtained from the culture collection of the Institute of Microbiology, Rutgers University, N.J.

Stock cultures were maintained on glucose–asparagine–salts agar slants; conidial suspensions rubbed off the surface of these slants into 1:5000 Tween 20 solution served as inocula. Shake cultures were incubated at ca. 27° C in a glucose–yeast extract medium and the mycelium harvested by filtration and washed with tap water and distilled water.

The mycelium was suspended in saline and to this was added one-fifth volume of 0.1 *M* phosphate buffer (pH 7.0 or 7.4). This suspension was mixed with an equal volume of 1 *M* sucrose and placed in a 37° C bath. Lysozyme (Nutritional Biochemicals Corp.) was added as a dry powder and

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dissolved by gentle rocking of the tube. In these experiments the concentrations of lysozyme used varied from 0.5 to 1 mg per ml of reaction mixture. Incubation was continued with occasional gentle mixing for about one hour.

After incubation the tubes were removed from the bath and allowed to stand at room temperature to permit the unlysed mycelium to settle out. The turbid supernate was poured through a thin layer of cotton batting wetted with 0.5 *M* sucrose and the filtrate then centrifuged at 3500 × *g* for 20 minutes.

Preparation of the antisera used in this work is described elsewhere (2). Further experimental procedures will be described in the appropriate sections.

Results

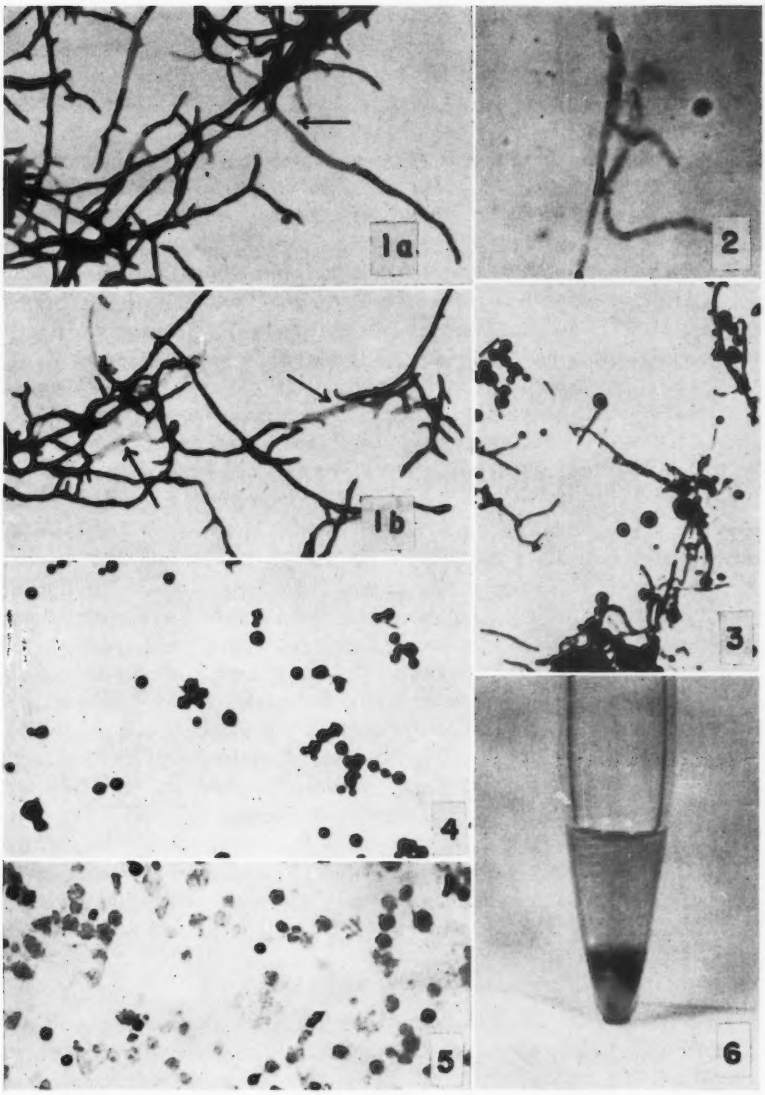
Observation of the digestion mixture showed the gradual disappearance of many of the mycelial clumps and the concomitant formation of numerous globular bodies ("protoplasts") in the suspending fluid. Figures 1 to 5 show the appearance of smears made from the reaction mixture. The most extensive conversion of hyphae to "protoplasts" occurred with 19- to 24-hour mycelium harvested from flasks which had received heavy conidial inocula so that the growth was mainly in the form of small clumps. Generally 48-hour mycelium pellets were completely refractory to lysozyme.

In many cases the reaction mixture became extremely viscous and it was necessary to dilute with 0.5 *M* sucrose before attempting the filtration. After centrifugation two discrete layers of sediment were generally observed, a bottom creamy-white layer consisting mainly of "protoplasts" and some mycelial fragments and an overlying layer (the color depending on the species used) comprised of mycelial fragments among which were entrapped additional "protoplasts". The supernatant fluid (extraprotoplastic lysate) was essentially free of formed elements. This centrifugal separation is shown in Fig. 6. The lower "protoplast" layer could be freed of most of the contaminating residual mycelium by resuspension in 0.5 *M* sucrose, centrifugation, and removal of the top layer.

With the experimental conditions employed *S. fradiae*, *S. olivaceous*, *S. scabies*, *S. lavendulae*, and *S. griseus* gave rise to large numbers of "protoplasts". "Protoplasts" could be formed from *S. ipomoeae* and *S. albus* but the yield was considerably lower. It should, however, be noted that efforts were directed mainly to establishing conditions most suitable for the lytic process as applied to *S. scabies*; these conditions were probably not optimum for all the other species examined.

When samples of "protoplasts" were sedimented and resuspended in sucrose solutions ranging from 0.1 to 0.8 *M* it was found that at concentration below 0.4 *M* many of the structures disappeared, presumably disrupted by osmotic shock. To avoid this, most manipulations were carried out in 0.5 *M* sucrose.

Salton (9, 10) has specified that in order to designate such lysozyme-induced structures as true protoplasts it should be shown that the cell wall has been removed completely. Two experimental approaches were employed in order to determine if complete removal had occurred in the present work.



FIGS. 1 to 5. *Streptomyces* spp.; crystal violet stained smears.
FIGS. 1*a* and 1*b*. *S. scabies*, isolate PA4, 24-hour shaken culture; arrows denote septa in apparently empty hyphae. FIG. 2. Same culture after 10 minutes' exposure to lysozyme; protoplasm rounding up inside partially lysed cell walls. FIG. 3. *S. fradiae* after 1 hour exposure to lysozyme; mixture of "protoplasts" and hyphae. FIG. 4. *S. fradiae*; "protoplasts" separated from hyphae by centrifugation. FIG. 5. *Streptomyces* spp.; "protoplast" preparation after storage; note ghost-like forms.
FIG. 6. Appearance of lysozyme-treated mixture after centrifugation.

A comparison was made between the original mycelium and the "protoplasts": (*a*) in their ability to adsorb phage and (*b*) in their serological characteristics.

The first stage in the infection of host cells by bacteriophage is the adsorption of phage particles to the host, apparently at receptor sites on the cell wall. Removal of the cell wall of the host should then result in its failure to adsorb phage. To test this hypothesis with *Streptomyces* "protoplasts", their ability to adsorb two actinophages was determined. Packed, intact mycelium, an approximately equal volume of packed "protoplasts", and extraprotoplastic lysate, both from the phage host *S. scabies* and from the non-susceptible culture *S. fradiae*, were added to phage suspensions in a medium of suitable osmotic strength and the per cent adsorption determined after 1 hour. Marked adsorption (70%) occurred only with the mycelium of *S. scabies*. A small amount (10%) of one of the viruses was adsorbed on *S. scabies* "protoplasts"; however, this was judged to be non-specific in nature since a similar amount of adsorption (15%) was observed on "protoplasts" and on intact mycelium of the non-susceptible *S. fradiae*. No marked effects on the virus titers were exhibited by the extraprotoplastic lysates. These data strongly suggest that lysozyme had effectively removed at least that portion of the cell wall containing phage receptor sites.

Vennes and Gerhardt (11) have shown that the injection of whole cells of *B. megaterium* into rabbits caused the formation of antibodies directed against cell wall antigens and protoplast membrane antigens. By serum absorption these authors showed that these surfaces were serologically distinct; however, in the present work it was not possible to demonstrate such differences with either *S. scabies* or *S. fradiae*. Absorption of anti-*fradiae* serum with washed intact mycelial clumps removed the "protoplast" agglutinins from the serum. In addition, the absorption of antisera (*S. scabies* and. *S fradiae*) with homologous washed "protoplasts" removed agglutinins for rabbit red blood cells sensitized with an "extraprotoplastic" antigen, presumably a cell wall substance (2). Both of these observations indicated that there was some cell wall antigen which was also present on the "protoplast" surface.

Preliminary experiments have shown that the transaminase and dehydrogenase activities of homogenates and sonic extracts of *S. scabies* (1) can occur also with the "protoplasts". A continuation of these studies is planned.

Discussion

In *B. megaterium* the formation of protoplasts from the vegetative cells is a quantitative process, each cell giving rise to one protoplast (12). Fitz-James has observed that elongated cells of *B. megaterium*, just prior to division, may give rise to two protoplasts but when this does occur there is always evidence for the initiation of septum formation (4, 5). At present there is some debate as to whether the mycelium of *Streptomyces* is septate (6) or coenocytic (3). For those species studied in the present work the fact that protoplast-like structures are formed would suggest that the protoplasm must have been present as at least potentially discrete "pockets" in the original hyphae.

The mycelial strands shown in Fig. 2 strongly support this view as do the apparent cell walls shown in Fig. 1. In some species however, this septation is not an obvious characteristic and might explain the low "protoplast" yield from some species.

The fact that older mycelium was refractory to lysis is similar to the findings with bacterial cells acted on by lysozyme (12). It is interesting to speculate whether an extension of the work of Romano and others (7, 8) would indicate some qualitative change in cell wall structure with age.

Osmotically sensitive protoplast-like structures have been produced from microorganisms by a variety of treatments. The present work extends these observations to include microorganisms with a mycelial growth habit. Although the data obtained from virus adsorption experiments indicate that "protoplasts" of the two *Streptomyces* species studied do not possess the phage-receptor component of the cell wall, the serological observations indicate that there may be some cell wall antigen(s) still present on the "protoplasts". Despite the fact that the properties described do not establish these structures as true protoplasts by the criteria of Salton (9, 10) it is obvious that lysozyme has removed from the mycelium that cell wall constituent responsible for structural rigidity and hyphal continuity.

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NOTES

EFFECTS OF TRICHOTHECIN ON SOME VIRUSES TRANSMITTED BY APHIDS[1]

R. H. E. Bradley and J. P. MacKinnon

Trichothecin is an antifungal substance from *Trichothecium roseum* Link ex Fr. (3). Freeman and Gill (2) showed it to be the isocrotyl ester ($C_{19}H_{24}O_5$) of the ketonic alcohol trichothecolone ($C_{15}H_{20}O_4$). It has been shown (1) that trichothecin prevents systemic infection of tobacco plants inoculated with potato virus Y by aphids. We have tested it against five more aphid-borne viruses, two of which persist in the vector.

Table I shows the results of four experiments in which we used methods similar to those described previously (1). The test plants were three-leaf seedlings of *Physalis floridana* Rydb. and white burley tobacco (*Nicotiana tabacum* L.). We sprayed five plants of each species with a solution of trichothecin, and five other plants with water to serve as controls. The concentrations of trichothecin were such that they caused little or no visible damage to the plants. About two hours after the plants were sprayed, when they were dry or nearly so, we transferred aphids to them from virus-infected plants. Two aphids were placed on each plant except in experiment 2, when one aphid was used. The aphids, wingless *Myzus persicae* (Sulz.), had had one of the following opportunities of acquiring viruses: several days on 'Chippewa' potato infected with the leaf-roll virus; 2 days on *P. floridana* infected with turnip-latent virus (4); or 2 minutes on tobacco infected with cucumber mosaic, henbane mosaic, or tobacco etch viruses. The aphids were removed after they had been on the test plants overnight. In experiment 4 we sprayed the plants with trichothecin a second time immediately after removing the aphids; this spraying was done to increase the possibility of trichothecin having an effect on the two viruses that persist in *M. persicae*. The first three experiments were done 8 times, the fourth 10 times.

Aphids transmit cucumber mosaic, henbane mosaic, potato Y, and tobacco etch viruses in apparently the same way. They do so most readily when they probe superficially first into infected and then into susceptible plants, and they cease to transmit these viruses soon after they leave the source. Table I shows that trichothecin prevented most systemic infections of all but the first of these four viruses. Why the effect on cucumber mosaic virus was so much less is not known; there may be differences in the method of its aphid transmission which have not been recognized, or it may be less susceptible to the effects of trichothecin.

[1]Contribution No. 3830, Entomology Division, and No. 1703, Botany and Plant Pathology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.

TABLE I

EFFECT OF TRICHOTHECIN ON SOME VIRUSES TRANSMITTED BY APHIDS

Expt. No.	Virus	Test plant	Concentration, p.p.m.	Plants sprayed		Plants infected*	
				Before inoculation	After inoculation	Treated	Untreated
1	Cucumber mosaic	Tobacco	3	Yes	No	12	17
2	Henbane mosaic	Tobacco	3	Yes	No	0	16
3	Tobacco etch	Tobacco	3	Yes	No	5	23
4	Potato Y	*P. floridana*	25	Yes	Yes	4	19
	Turnip-latent	*P. floridana*	25	Yes	Yes	14	26†
	Potato leaf-roll	*P. floridana*	25	Yes	Yes	26	29

*Plants infected out of 40 in experiments 1, 2, and 3; out of 50 in experiment 4.
†Significant difference between number of treated and untreated plants. $\chi^2 = 5.04$; P .05 = 3.84.

In experiment 4 we compared the effects of trichothecin on potato virus Y and the two viruses that persist in *M. persicae*, namely turnip-latent virus and potato leaf-roll virus. To test these in a common host, *P. floridana* was used as the test plant. Table I shows that trichothecin prevented most systemic infections of potato virus Y, nearly half of those of turnip-latent virus, but scarecely any of those of potato leaf-roll virus. Trichothecin seemed not to deter aphids from feeding; and if it had, one would expect fewer transmissions of leaf-roll virus to trichothecin-treated plants. We did not expect that trichothecin would stop plants from becoming infected with turnip-latent or potato leaf-roll viruses. These two viruses, unlike the others, are thought to be transmitted after aphids penetrate well beyond the superficial tissues. Presumably it would be more difficult to affect these viruses by spraying a substance on the leaf surfaces. The unexpected result with turnip-latent virus is further evidence that this approach to the control of aphid-borne viruses warrants further study.

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